FINAL REPORT

USING NEEM EXTRACTS TO CONTROL PESTS OF THE HONEY BEE

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Neem Extracts in Honey Bee Pest Management

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Colonies of the honey bee are susceptible to a number of pests and diseases whose damage has serious negative economic impact for both the beekeeping industry and agriculture. These pest problems have been further aggravated with the recent introduction of two parasitic mites in North America, the varroa (*Varroa jacobsoni*) and tracheal (*Acarapis woodi*) mites. Together these two mites have severely reduced the number of healthy colonies available for beekeeping and pollination, a service valued at over $20 billion Canadian in North America alone.

Beekeepers have become increasingly dependent on the use of pesticides and antibiotics to combat bee pests, leading to several problems including increased treatment and labor costs, toxicological hazards to beekeepers and bees, and risks of contaminating hive products. Moreover, current management of these pests depends on only one or two control agents, leaving beekeepers vulnerable to the evolution of resistance.

Our research focussed on developing a new agent, neem, for simultaneously controlling multiple honey bee pests, with low toxicological effects on beekeepers and bees and without pesticide residues in honey or wax. Beekeepers world-wide, including Saskatchewan, would derive economic benefit from an effective neem-based control technology through lower treatment and labour costs and higher yields of honey and wax products accrued from control of the major honey bee pests.

The most important finding of this project was that neem oil has potential to control varroa, tracheal mites, and the bacterial brood disease American Foul Brood, although not the fungal parasite chalkbrood. The details of this research can be found in the enclosed three manuscripts, all of which will be published in scientific journals shortly. Below is a point-form summary of the principal research results from these studies:

- We developed a unique and rapid bioassay screening system to quickly evaluate new potential miticides, including neem but also other substances such as essential oils.

- We also developed methods to quickly screen miticides against mite parasites and bacterial and fungal diseases.

- Laboratory bioassays indicated that neem had good potential to control varroa, tracheal mites, and American Foul Brood. In particular, neem oil was most effective, more so than a refined neem azadarachtin-mix fraction.

- Other oils also showed some potential for mite control.

- Field evaluation of neem and canola oil suggested that neem oil sprayed on bees during summers could be effective for both varroa and tracheal mite control.

Currently, the principal student involved in these projects, Adony Melathopolous, is pursuing funding to take this work towards commercial applications. We hope to see further product development commence shortly, leading to a new product useful for beekeepers in controlling mites and other diseases. While neem is effective, its formulation and release device will be critical, and it is this stage at which research is needed to complete product development.

Reporting of Results

We have made considerable effort to present the results of our findings to beekeeping audiences, in addition to our scientific publications. Reports of this project have been published regularly in the Saskatchewan beekeeping newsletter put out by Saskatchewan Agriculture, as well as in *Hive Lights*, the newsletter of the Canadian Honey Council.

In addition, we have traveled extensively to present results at beekeeping meetings, including annual attendance and talks presented at the Saskatchewan Beekeepers Association meeting, as well as the national meeting of the Canadian Honey Council. Further, these results have been presented at local, national, and international meetings. Just this year, I have already spoken in Saskatchewan, Manitoba, Illinois, and England to present these findings to beekeeping audiences. Finally, papers were given at the international Apimondia meeting in September 1999, held in Vancouver, at which 3000 people were in attendance, as well as an international neem conference.

In summary, I believe this project accomplished its objectives, and has provided some important findings for bee management. We have been diligent, indeed enthusiastic, about interacting with beekeepers around the world to distribute these results in a beekeeper-friendly fashion, in addition to the more "traditional" route of publishing in scientific journals. We greatly appreciate the support provided by the Agri-Food Innovation Fund, and in our written and verbal presentations have been pleased to be able to acknowledge that important support.

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Apiculture and Social Insects

Field Evaluation of Neem and Canola Oil for the Selective Control of the Honey Bee (Hymenoptera: Apidae) Mite Parasites *Varroa jacobsoni* (Acari: Varroidae) and *Acarapis woodi* (Acari: Tarsonemidae)

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ABSTRACT Neem oil, neem extract (neem-aza), and canola oil were evaluated for the management of the honey bee mite parasites *Varroa jacobsoni* (Oudemans) and *Acarapis woodi* (Rennie) in field experiments. Spraying neem oil on bees was more effective at controlling *V. jacobsoni* than feeding oil in a sucrose-based matrix (patty), feeding neem-aza in syrup, or spraying canola oil. Neem oil sprays also protected susceptible bees from *A. woodi* infestation. Only neem oil provided *V. jacobsoni* control comparable to the known varroacide formic acid, but it was not as effective as the synthetic product Apistan (τ-fluvalinate). Neem oil was effective only when sprayed 6 times at 4 d intervals and not when applied 3 times at 8 d intervals. Neem oil spray treatments had no effect on adult honey bee populations, but treatments reduced the amount of sealed brood in colonies by 50% and caused queen loss at higher doses. Taken together, the results suggest that neem and canola oil show some promise for managing honey bee parasitic mites, but the negative effects of treatments to colonies and the lower efficacy against *V. jacobsoni* compared to synthetic acaricides may limit their usefulness to beekeepers.

KEY WORDS Acaricide, mite, neem, canola oil

The parasitic *Varroa* (*Varroa jacobsoni* (Oudemans)) and tracheal (*Acarapis woodi* (Rennie)) mites threaten the honey bee (*Apis mellifera* L.) industry worldwide. These two mites have severely reduced the number of healthy colonies (Matheson 1994, Kraus and Page 1995, Finley et al. 1997) available for beekeeping and pollination, a service valued at over $10 billion annually in North America alone (Robinson et al. 1989, CAPA 1995). Honey bees are highly susceptible to *Varroa*, and colony death follows 1-2 consecutive years of infestation (Martin et al. 1998, Downey et al. 1999). Damage caused by tracheal mites varies considerably with environmental conditions (Eischen 1987, Eischen et al. 1989, Harbo 1993, Frazier et al. 1995) and colony genetics (Page and Gary 1990), but epidemic colony losses of up to 70% and severely reduced colony productivity have been reported (Eischen 1987, Otis et al. 1988, Eischen et al. 1989). Although it is unclear if Old World honey bee stocks have adapted to tracheal mites (Bailey and Ball 1992, Frazier et al. 1995), dual infestation with *Varroa* may result in rapid colony decline even in regions where tracheal mites are not considered a problem on their own (Downey et al. 1999).

At present, the management of *Varroa* relies on the synthetic acaricides τ-fluvalinate and flumethrin (pyrethroids) (Ferrer-Dufol et al. 1991), amitraz (amidine) (Herbert et al. 1988b, Wilson et al. 1998), and coumaphos (organophosphorothioate) (Milani and Iob 1998, Ellis et al. 1998, Wilson et al. 1998). *Varroa* have developed tolerance to these synthetic acaricides in many areas (reviewed in Milani 1999), and acaricide residues have appeared in honey and wax products (reviewed in Wallner 1999).

Tracheal mites, by contrast, have been successfully managed in North America without synthetic acaricides. Management of tracheal mites relies exclusively on

naturally derived acaricides, specifically, menthol (Cox et al. 1986, Delaplane 1992), formic acid (Hoppe et al. 1989, Feldlaufer et al. 1997) and vegetable seed oil (Delaplane 1992, Sammataro et al. 1994, Calderone and Shimanuki 1995). Use of these natural products has largely overcome the problems associated with synthetic acaricides. To date there have been no cases of acaricide resistance, and although residues have been found in honey and wax, they are generally less persistent than the synthetic products used to manage *Varroa* (Li et al. 1993, Wallner 1999). Although natural acaricides have also been screened for activity against *Varroa*, and include organic acids (Hoppe et al. 1989, Kraus and Berg 1994, Feldlaufer et al. 1997) and thymol (reviewed Imdorf et al. 1999), they are not as effective or consistent as synthetic products. As a consequence beekeepers continue to rely heavily on synthetic formulations for the control of *Varroa*.

Oil from the seeds of the Indian neem tree, *Azadirachta indica* A. Juss., and the canola plant, *Brassica napus* L., offer a novel and safe method for managing honey bee mites. Both neem and canola oil applied topically to bees are effective at killing phoretic *Varroa* (Melathopoulos et al. 2000). Furthermore, dispersal of tracheal mites to new bee hosts is disrupted with exposure to vegetable (Smith et al. 1991, Melathopoulos et al. 2000) and neem oil (Melathopoulos et al. 2000). Vegetable oils formulated with sucrose as a patty significantly limit population growth of tracheal mites in honey bee colonies (Deleplane 1992, Sammataro et al. 1994, Calderone and Shimanuki 1995). Preliminary results suggest that oil products sprayed on bees result in >90% mortality of *Varroa* under field conditions (Le Conte et al. 1998). Neem oil also has low environmental persistence (Sundaram and Curry 1994), does not induce resistance readily (Feng and Isman 1995), and is relatively non-toxic to mammals (Larson 1989, Jacobson 1995).

The objective of this study was to evaluate different application methods and rates of neem and canola oil for effective and simultaneous control of *Varroa* and tracheal mites in the field.

Materials and Methods

Chemical Compounds. Cold pressed neem seed kernel oil (neem oil) and unformulated azadirachtin-rich (10% azadirachtin wt : wt) neem seed insecticide (neem-aza) used in 1997 experiments were gifts from Neem International Enterprises Inc. (Surrey, British Columbia). Neem-aza was diluted in EtOH to 0.3% (wt : wt) for all experiments to ensure solubility in sucrose syrup solutions. Neem oil used for 1998 experiments was a gift from Trifolio-M GmbH (Lahnau, Germany). Debitterized neem oil was made by making 5 successive washes of neem oil with EtOH (reagent grade, Sigma, Oakville, Ontario) at 40°C. The wash provided a 53% yield of debitterized oil from the crude oil. Canola seed oil was obtained from Lucerne Foods Ltd. (Vancouver, British Columbia). Tween-20 emulsifier was used to emulsify oil treatments in water for spray treatments and was obtained from Sigma. Acaricide standards were Apistan™ (*Varroa*), a controlled release plastic strip containing 10% (wt:wt) τ-fluvalinate (Wellmark International, Dallas, TX) and 65% formic acid (*Varroa* and tracheal mite) (Medivet Pharmaceuticals, High River, Alberta).

Bees and Mites. Colonies used in the experiment were established through division of larger colonies with heavy infestations of either *Varroa* or tracheal mites. Limited treatment with the acaricides Apistan and formic acid and periodic addition of healthy and mite-infested honey bee adults, larvae, and pupae was conducted >1 mo prior to dividing colonies for experiments, to ensure adequate mite levels, prevent colony

death, and maintain infestation with only one mite species. For all experiments, colonies had a mated queen for >1 wk prior to treatment, with the exception of experiment 5 where a queen mandibular pheromone surrogate was supplied in lieu of a queen (Bee Boost, PheroTech Inc., Delta, British Columbia). All colonies were housed on full-size Langstroth hive bodies (supers), each consisting of 10 moveable frames of comb and enclosing a space of approximately 40 liters (Graham 1997). A full frame of comb holds approximately 2400 adults (Burgett and Burikam 1985), 15,000 eggs, larvae or pupae, or 4 kg of honey (Winston 1987). Colonies used in experiments consisted of one super, two stacked supers or a half-sized, nucleus super.

The honey bees used in experiments could not be classified into a specific race of *A. mellifera* because stocks of several different races have been imported to and interbred within the region (Sheppard 1989). Two distinct haplotypes of *V. jacobsoni* exist in North American, one originating from the Russian Far East and the other from Japan/Thailand (Guzman et al. 1999). Genetic analysis of *V. jacobsoni* specimens obtained from British Columbia suggest mites in our study area were Russian (Guzman et al. 1999).

Experiments. Experiments consisted of treating colonies infested with either *Varroa* or tracheal mites with neem, canola oil, or known acaricides, and assessing their ability to control the mites without harming the resident bees.

Treatments containing neem or canola oil were either 1) fed to bees in sucrose patties or 2) sprayed directly on bees. Each sucrose patty consisted of 25 g of oil and 75 g of sucrose mixed between wax paper and placed directly across the top of the frames (Calderone and Shimanuki 1995). Oil was sprayed directly on bees as an emulsion in

water, using 2% (wt:wt) Tween-20 as the emulsifier. Emulsions were sprayed at a rate of 20 ml per full frame of bees at approximately 400 kPa using a backpack sprayer with a cone nozzle (SVV™ Survivor Sprayer, SP Systems, CA). Treatments were applied by creating a 5-cm gap between each frame and running the sprayer nozzle across the length and depth of the comb, providing uniform coverage of the bees on the adjacent frame faces. This method of spraying evenly distributed the spray throughout the colony without the need to remove frames individually. The emulsion was manually agitated prior to spraying each colony. Unless otherwise specified, spray treatments were applied 6 times at 4 d intervals. Neem-aza dissolved in EtOH was fed to bees mixed with 2 M sucrose syrup using inverted 4-liter bucket feeders (Graham 1997). Colonies not treated with neem-aza were fed untreated syrup. Formic acid (40 ml) was applied to each colony using absorbent Mite Wipe™ pads (Medivet Pharmaceuticals). The pads were placed on the top bars of the frames. Pads loaded with formic acid were replaced six times at 4 d intervals, unless otherwise specified. Apistan strips were suspended in the center of the brood chamber, 1 strip per 5 full frames of bees. Consumption of syrup and patties during the treatment was determined by subtracting the start weight of full feeders and patties from the weight at the end of the treatment.

Varroa killed during experiments were collected on 30 × 40 cm cardboard traps coated with Sticky-Stuff adhesive (Olsen, Medina, OH) which were placed on the bottom board of the hive (Calderone and Spivak 1995). Bees were restricted from contacting the adhesive surface with 6.4 mm wide mesh. Adhesive boards were replaced at 4--8 d intervals to prevent the surface from becoming saturated with debris. Treatments were evaluated by placing Apistan strips into each colony to kill remaining mites. *A. woodi*

infestation among adult bees was determined by dissecting the main prothoracic tracheal trunks using techniques adapted from Eischen et al. (1987).

The impact of treatments on colonies was determined by comparing adult worker and sealed brood (prepupal and pupal stages) populations prior to and following treatments. Populations were estimated by placing a piece of clear plastic with an inscribed 5 × 5 cm grid over each frame side and counting the number of grids covered with workers or brood. Loss of queens from colonies following treatment was determined when queens could not be located after careful inspection of combs and 1-d old eggs were absent.

Experiment 1. Evaluating Different Methods of Administering Neem for the Control of Varroa. The experiment was conducted on blooming clover fields adjacent to the Ministry of Agriculture and Food complex in Abbotsford, British Columbia, between 5 and 30 August 1997. The weather throughout the experiment was sunny and warm (18-35°C daytime temperatures). Experimental colonies consisted of approximately 1 frame of mixed eggs, larvae, and pupae and 2 frames of honey and pollen provisions. Enough workers were provided for 2.5 frames to be covered with.

The experiment compared oral and spray treatments of neem to the acaricide standard formic acid for *Varroa* control (Table 1). Treatments were applied first on 5 August. Apistan was placed in all colonies on 29 August for 1 d to estimate of the relative number of *Varroa* remaining in the colony following treatment.

Experiment 2. Evaluation of Fall Neem Treatments for Varroa *Control.* The experiment was conducted on grass pasture located in Fort Langley, British Columbia, between 12 October and 30 November 1997. The weather throughout was partly sunny

and cool (8-15°C daytime temperatures). Colonies consisted of 3 frames of mixed eggs, larvae, and pupae and 4 frames of honey and pollen. Approximately 6 frames of bees were present in each colony.

This experiment evaluated neem treatments under fall conditions, when *Varroa* are most susceptible to acaricide treatment owing to declining amounts of sealed brood. Higher rates of neem-aza syrup and debitterized neem oil were used (Table 1) based on the results of experiment 1. Treatments were applied first on 13 October. All colonies were treated with Apistan between 2 and 30 November to estimate the number *Varroa* remaining in the colony following treatment.

Experiment 3. Comparison Between Neem and Canola Oil Sprays for the Control of Varroa. Colonies were established in Bradner, British Columbia, and the experiment ran between 15 May and 3 July 1998. Conditions were sunny and warm with daytime temperatures ranging between 12 and 25°C. Colonies consisted of approximately 2 frames of eggs, larvae, and pupae, 2 frames of honey and pollen, and 2 frames of adult worker bees.

The experiment tested the hypothesis that canola oil sprays are as effective as neem oil at controlling *Varroa* (Table 1). Treatments were first applied on 15 May and colonies were all treated with Apistan between 8 June and 3 July. Adult bee and sealed brood populations were determined prior to and following treatments on 15 May and 8 June, respectively.

Experiment 4. Effect of Spray Frequency and Application Method on Varroa *Control.* Colonies were established adjacent raspberry fields at the Abbotsford Municipal Airport, British Columbia, between 23 July and 10 October 1998. Daily temperatures

were between 18 and 30°C and conditions were warm and sunny. Colonies had approximately 2 frames of mixed eggs, larvae, and pupae, 2 frames of honey and pollen, and 1 frame of adult worker bees.

Different timing and methods of spraying neem oil and doses of canola oil were evaluated for *Varroa* in this experiment (Table 1). Two different spray timings were compared; 6 spray applications applied at 4 d intervals, which was the frequency used in all previous experiments, and 3 applications at 8 d intervals. A more rapid method of applying sprays, called the top spray method, also was evaluated. The top spray method involved spraying between frames from the top of the colony without creating an additional gap.

Treatments began on 23 July, continued for 24 d, and then all colonies were treated with Apistan between 16 September and October 10 to estimate the size of the remaining *Varroa* population. Adult bee and sealed brood populations were determined prior to and following treatments on 23 July and 16 September, respectively.

Experiment 5. Inhibition of Tracheal Mite Host Migration Using Neem and Canola Oil Sprays. Colonies were established on the campus of Simon Fraser University, British Columbia, between 22 March and 1 April 1998. Daily temperatures were 8--12°C and conditions rainy and cool. Colonies were established in nucleus sized supers. Colonies were queenless, but were supplemented with queen mandibular pheromone (BeeBoost), and had approximately 0.5 frame of mixed eggs, larvae, and pupae, 1 frame of honey and pollen, and 1 frame of adult worker bees.

Newly eclosed workers are the most susceptible to becoming infested by tracheal mites searching for new hosts (Gary et al. 1989). Consequently, newly eclosed workers

were added to each colony on 24 March, to determine if oil treatments could protect workers from becoming infected with tracheal mites, using techniques adapted from Smith et al. (1990). Newly eclosed workers were obtained from frames of sealed brood emerged in an incubator at 32°C for 18 h. An enamel paint mark (Testors, Weston, Ontario) on the tip of the abdomen distinguished these bees from other bees in the colony. The paint marks themselves do not influence mite host location (Smith et al. 1990). Exactly 50 newly eclosed workers were introduced directly into each colony after acclimatizing for 4 h in mesh cages placed on the top of the frames.

The sucrose patty treatment was applied on 22 March, 48 h prior to the introduction of the marked workers. Remaining treatments (Table 1) were applied 8 h after marked workers were introduced. Formic acid and oil sprays were reapplied on 28 and 31 March. Marked bees were recovered on 1 April, preserved in EtOH, and 30 workers per colony were dissected to determine how many were infested.

Data Analysis. Varroa. The proportion of *Varroa* in colonies killed during the treatment (P_{Varroa}) was estimated using the formula, $P_{Varroa} = M_{treatment} / (M_{treatment} + M_{evaluation})$, where $M_{treatment}$ is mortality due to treatment (estimated from the total number of mites caught on adhesive boards during the treatment period) and $M_{evaluation}$ was the number of mites not killed by the treatment (estimated from the number of mites caught with Apistan following treatment). The hypotheses that treatment had no effect on 1) sealed brood populations, 2) adult worker populations, or 3) arcsine square-root transformed treatment efficacy (P_{Varroa}) (Sokal and Rolf 1981) were tested with analysis of variance (ANOVA) (SAS Institute 1997). Treatment differences were compared using Tukey-Kramer Honest Significant Differences (HSD) (SAS Institute 1997). Treatment

effects on colony queen loss were tested using a modified Student-Neuman-Keuls test (Steel and Torrie 1980, Jones 1984). The hypotheses that colony consumption of 1) neem treated syrup was different from untreated syrup or 2) neem oil patties was different from canola oil patties were tested with ANOVA (SAS Institute 1997).

Tracheal Mite. The hypotheses that treatment influenced either the 1) number of marked bees recovered from each colony or 2) arcsine square-root transformed proportion of bees infested with tracheal mites were tested using ANOVA. Treatment differences were compared using Tukey-Kramer HSD (SAS Institute 1997).

Results

Experiment 1. Evaluating Different Methods of Administering Neem for the Control of Varroa. Treatment effects resulted in significant *Varroa* mortality ($F = 6.05$; df = 7, 35; $P < 0.01$). Analysis of means revealed that mortality in colonies treated with formic acid was greater than in all of the other groups, except the 10% neem oil spray group (Fig. 1 A).

Experiment 2. Evaluation of Fall Neem Treatments for Varroa *Control.* Treatment effects resulted in significant *Varroa* mortality ($F = 79.7$; df = 5, 42; $P < 0.01$). Apistan provided the highest degree of control, significantly greater than that in all other groups (Fig. 1 B). The 10% neem oil spray and formic acid treatments provided similar levels of control, both of which were greater than in the syrup, patty and control groups. There were no differences among the syrup, patty and control groups.

Methods of administering neem that relied on bees' feeding, such as in sucrose patties or syrup, were not consumed readily (Figure 2, 3). Although debitterizing neem oil increased the palatability of patties to bees, it did not result in significant *Varroa*

control (Figure 1 B).

Experiment 3. Comparison Between Neem and Canola Oil Sprays for the Control of Varroa. Treatment effects resulted in significant *Varroa* mortality ($F = 41.2$; df = 5, 37; $P < 0.01$). As was the case in the previous experiment, Apistan provided the highest level of mite control, exceeding that of the other treatments significantly (Fig. 1 C). Formic acid, 10% canola oil spray and 5 and 10% neem oil spray treatments provided significant control compared to colonies remaining untreated, however 10% canola oil was not as effective as formic acid.

Although worker and sealed brood populations were not different among groups at the beginning of the experiment ($F = 0.22$; df = 5, 40; $P = 0.95$), 5 and 10% neem oil spray and formic acid groups had <50% the sealed brood of the untreated group following treatment ($F = 5.42$; df = 5, 35; $P < 0.01$) (Fig. 4). Furthermore, 50% of colonies treated with 10% neem oil spray lost their queen, compared to no queen loss in untreated colonies (Fig. 5). There was, however, no treatment differences in adult worker population following treatment ($F = 2.10$; df = 5, 38; $P = 0.09$).

Experiment 4. Effect of Spray Frequency and Application Method on Varroa *Control.* Treatment effects resulted in significant *Varroa* mortality ($F = 8.15$; df = 5, 42; $P < 0.01$). While top spray treatments were as effective as the more labor intensive method of spraying between frames, reducing the frequency of neem oil sprays from 6 treatments at 4 d intervals to 3 treatments at 8 d intervals rendered neem oil ineffective (Fig. 1D).

Although there was no difference in sealed brood population among groups before treatment ($F = 0.92$; df = 6, 48; $P = 0.49$), significant difference existed following

treatment ($F = 3.99$; df = 6, 48; $P < 0.01$). Treatment with 6 × 10% neem oil applied frame by frame or by the top method resulted in significant reduction in sealed brood populations compared to untreated colonies (Fig. 4).

Adult worker population was not different among treatments either before ($F = 1.16$; df = 6, 47; $P = 0.35$) or following treatment ($F = 0.92$; df = 6, 48; $P = 0.49$). Furthermore, queen loss among colonies was not affected by treatment (Student-Neuman-Keuls, $P < 0.05$).

Experiment 5. Inhibition of Tracheal Mite Host Migration Using Neem and Canola Oil Sprays. Treatment effects resulted in significant reduction in tracheal mite host transfer ($F = 7.23$; df = 4, 35; $P < 0.01$). Only neem and canola oil sprays provided comparable control to formic acid and both had significantly lower incidences of tracheal mite infestation compared to untreated groups (Fig. 6). Although canola oil formulated in a patty provided comparable control to neem and canola spray treatments, it did not provide significant protection from tracheal mite infestation compared to untreated groups. None of the treatments influenced the number of marked target workers recovered following treatment ($F = 1.10$; df = 4, 35; $P = 0.373$), which averaged 46.0 $\pm$ 3.7, or approximately 92% of the original number introduced.

Discussion

Our field experiments demonstrated that neem and canola oil sprayed on bees provide moderate control of *Varroa* and protection from infestation by tracheal mites, but with some negative impact on colony brood rearing and queen survival using neem oil. No study has previously investigated neem oil in the field as a control agent for honey bee mites and only one other published study has evaluated spraying oils to manage any

honey bee pest (Le Conte et al. 1998).

Levels of *Varroa* mortality observed with oil spray treatments varied between experiments. One source of variation may be differences in the methods used to estimate efficacy. Experiment 1 evaluated treatment efficacy following 1 d of treatment with Apistan, whereas all other experiments used 24 d of Apistan treatment. The short post-treatment evaluation with Apistan in experiment 1 likely underestimated the number of total mites in the colony for calculations of treatment efficacy. Among the remaining experiments, treatment efficacy for neem oil sprayed 6 times at 4 d intervals varied between 50--80% and for canola oil between 30--70%. Treatments appeared to be least effective during the summer. Acaricides work best against *Varroa* in early spring and fall because at these times colony brood rearing has not reached its peak, resulting in most of the mite population residing on adult hosts where they are more accessible to treatment (Koeniger and Fuchs 1989, Calderone and Spivak 1995, Calderone et al. 1997, Le Conte et al. 1998).

The levels of treatment efficacy obtained for canola oil were considerably lower than the 95% *Varroa* mortality reported previously for broodless colonies (Le Conte et al. 1999), although comparable to the 57% observed when colonies had brood. In our study, canola oil only was evaluated during periods when colonies had brood present, and thus our results are consistent with those of Le Conte et al. (1998). Further, Le Conte et al. (1998) report efficacy approaching 100% following 3 applications of paraffin oil and emulsifier in colonies containing brood. The performance of paraffin oil and emulsifier far exceeded that of the best neem oil treatment in our experiments (June 1998; 80% *Varroa* mortality).

The ability of oil sprays to prevent tracheal mite host transfer was not entirely unexpected, as oil formulated in sucrose patties has already been shown to have this effect (Delaplane 1992, Sammataro *et al.* 1994, Calderone and Shimanuki 1995). What was surprising was that oil sprays provided comparable tracheal mite control to formic acid, a highly effective tracheal mite acaricide, whereas oil formulated as a patty did not. While oil spray treatments were not directly found to be more effective at preventing tracheal mite infestation than oil patties, it is possible that spray treatments exceed the control currently offered by patties, but differences were not detected in our experiment. A lack of significant difference may have arisen from focusing on mite host transfer to a cohort of susceptible workers, whose numbers were relatively low compared the total number of workers in the colony. Consequently, nonsignificant differences in mite infestation between oil spray and oil patty treatments may have translated to significant differences when assessed over the whole colony's mite population. Furthermore, patty and spray treatments were not evaluated beyond the treatment period, and consequently, it was unknown how mite populations would grow following treatment. For these reasons, further studies comparing tracheal mite populations between oil spray and patty treatments over the span of an entire season should be encouraged.

Although neem oil spray treatments had no effect on adult honey bee populations, they reduced the amount of sealed brood in colonies by 50% and caused significant loss of queens at a concentration of 10% in June 1998. Azadirachtin-enriched extracts of neem oil are acutely toxic to immature honey bees (Rembold et al. 1980) and have an LD_{50} for first instar worker larvae estimated at 37 µg/g azadirachtin (Naumann and Isman 1996). Consequently, reduced brood area may have been the result of poisoning with

azadirachtin. Formic acid also had a negative impact on brood area in experiment 3. Although previous studies have documented that formic acid does not impact brood survival or production (Westcott and Winston 1999), our experiment was performed in small colony units, which may be more sensitive to toxic effects of the vapor.

Lack of significant impact of neem oil on adult honey bee workers is consistent with laboratory results demonstrating that the LC_{70} for *V. jacobsoni* was 2--10 times lower than the LC_{30} of adult workers (Melathopoulos et al. 2000). The impact of neem oil treatments on colony queen loss is more difficult to explain, but may be the result of worker aggression towards the queen following treatment or departure of the queen from the colony. The latter hypothesis is supported by observations that queens would often emerge at the top of the colony a few minutes following treatment. The repulsive effect of neem oil on queens, however, was only observed with the Trifolio-M product used in 1998 and did not occur using the product produced by Neem International Enterprises in 1997. Possibly repulsive constituents in the oil vary between manufactures as a result of differences in the source seed or in manufacturing practices.

Although the material cost of spraying oil is small, the labor involved with spraying oil was higher compared to acaricides currently used by beekeepers. Both neem and canola oil cost approximately $1.25 per 10 frames of bees sprayed, which is marginally more than the cost of treatment with 65% formic acid but half the cost of treatment with Apistan. Treatment of 10 frames with Apistan takes approximately 30--40 sec and requires beekeepers to visit the apiary once to insert the strip and once to remove it. Formic acid treatment of 10 frames of bees takes under 30 seconds, but requires 5--6 application for moderate to high *Varroa* control. By contrast, oil sprays took

approximately 90 sec to apply per colony and required 6 applications. More efficient methods of applying treatments that reduce the time required per application and the need for reapplication is thus key to making the treatments economically viable for beekeepers.

Neem and canola oil show some promise for managing honey bee mites, but a number of obstacles remain, including moderate *Varroa* control compared to synthetic acaricides, unwanted side-effects among colonies treated with neem and labor intensive treatment methods. The future direction of this research should be to increase treatment efficacy through improved formulation and treatment methodology, to reduce the negative impacts of neem oil by formulation and removal of toxic ingredients, and the development of more efficient application technology.

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Table 1. Description of treatments, rate, and concentration applied to colonies in experiments

Exp	Mite Infestation	Date	Treatment (No. colonies)	Application Method (No. applications)	Rate	Concentration[a]
1	*Varroa*	Aug.	untreated (6)	-	-	-
		1997	neem-aza (5)	syrup feed	4 liter	3 ml / liter syrup
			neem-aza (5)	syrup feed	4 liter	9 ml / liter syrup
			neem oil (5)	sucrose patty	100 g	25%
			canola oil (6)	sucrose patty	100 g	25%
			neem oil (6)	spray (6 ×)	40-80 ml[b]	1%
			neem oil (5)	spray (6 ×)	40-80 ml[b]	10%
			formic acid (5)	Mite Wipe (6 ×)	30 ml	65%
2	*Varroa*	Oct.	untreated (9)	-	-	-
		1997	neem oil[c] (8)	sucrose patty	100 g	25%
			neem-aza (8)	syrup feed	4 liter	17 ml / liter syrup
			formic acid (8)	Mite Wipe (6 ×)	30 ml	65%
			neem oil (8)	spray (6 ×)	60-240 ml[b]	10%
			Apistan (7)	strip	1-2[d]	10%

Exp	Mite Infestation	Date	Treatment (No. colonies)	Application Method (No. applications)	Rate	Concentration[a]
3	*Varroa*	June	untreated (8)	-	-	-
		1998	canola oil (7)	spray (6 ×)	10-100 ml[b]	10%
			neem oil (8)	spray (6 ×)	10-100 ml[b]	5%
			neem oil (8)	spray (6 ×)	10-100 ml[b]	10%
			formic acid (7)	Mite Wipe (6 ×)	30 ml	65%
			Apistan (7)	strip	1-2[d]	10%
4	*Varroa*	Aug.	untreated (9)	-	-	-
		1998	emulsifier (8)	spray (6 ×)	30-140 ml[b]	2%
			canola oil (8)	spray (6 ×)	30-140 ml[b]	10%
			canola oil (9)	spray (6 ×)	30-140 ml[b]	20%
			neem oil (8)	spray (3 ×)	30-140 ml[b]	5%
			neem oil (8)	spray (6 ×)	30-140 ml[b]	5%
			neem oil (8)	top spray (6 ×)[e]	30-140 ml[b]	5%
5	tracheal mite	Apr.	untreated (8)	-	-	-
		1998	canola oil (8)	sucrose patty	100 g	25%
			canola oil (8)	spray (3 ×)	10-20 ml[b]	10%
			neem oil (8)	spray (3 ×)	10-20 ml[b]	10%
			formic acid (8)	Mite Wipe (3 ×)	30 ml	65%

[a] Doses expressed as a percentage are wt:wt.

[b] Spray volume adjusted to colony size with approximately 20 ml per full frame of bees.

[c] Debitterized neem oil.

[d] Number of strips adjusted to colony size with 1 strip for approximately 5 full frames of bees.

[e] Spray applied on top of frames.

Fig. 1. Mean (± SE) *Varroa* mortality following treatment with neem, canola oil, and neem-aza (Table 1; August 1997 (A), October 1997 (B), June 1998 (C), and August 1998 (D)). Treatments followed by the same lower-case letter indicate no significant difference in varroa mortality (transformed) (Tukey-Kramer HSD, $P = 0.05$).

Fig. 2. Mean (± SE) consumption of 100 g sucrose:oil (3:1) patties containing either crude neem oil, debitterized neem oil, or canola oil. Bars with the same letter indicate no significant difference in consumption (ANOVA, $P = 0.05$).

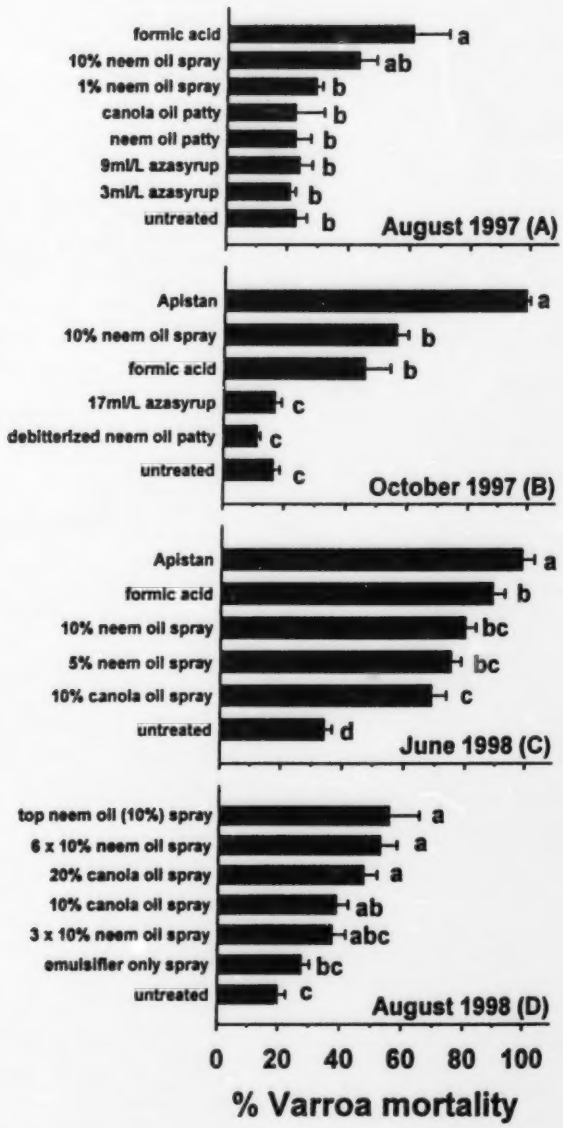
Fig. 3. Mean (± SE) consumption of 4.5 kg of untreated 2 M sucrose syrup or syrup treated with 17 ml / liter of 0.3% (wt : wt) neem-aza. Different letters above bars indicate treatment differences (ANOVA, $P = 0.05$).

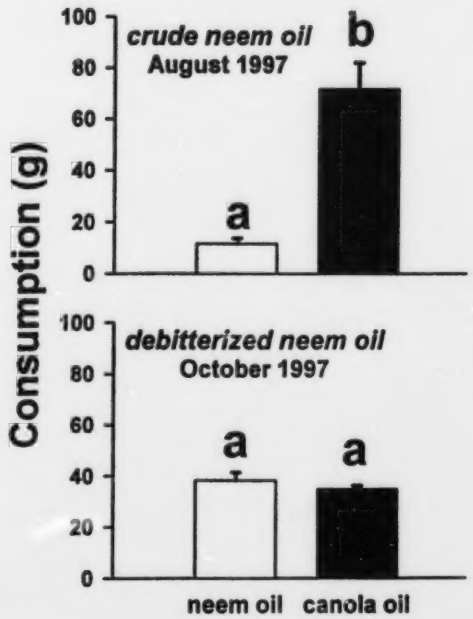
Fig. 4. Estimated mean (± SE) colony worker and sealed brood population before and after treatment with neem, canola oil, formic acid, and Apistan. Worker population and brood area in June 1998 and August 1998 were not different between treatments prior to the experiment (ANOVA, $P = 0.05$). The mean worker and brood populations prior to the experiment, across all treatments, were therefore pooled and are indicated by the gray vertical lines on each graph. Treatments followed by the same letter indicate no significant difference in population (Tukey-Kramer HSD, $P = 0.05$).

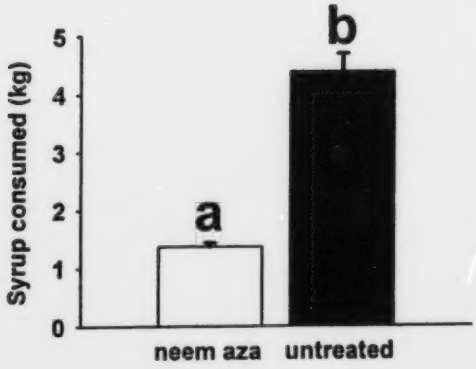
Figure 5. Proportion of colonies with queens remaining following treatment with neem, canola oil, formic acid, and Apistan (June 1998). Treatments followed by the same letter indicate no significant difference in queen loss (modified Student-Neuman-Keuls, $P = 0.05$).

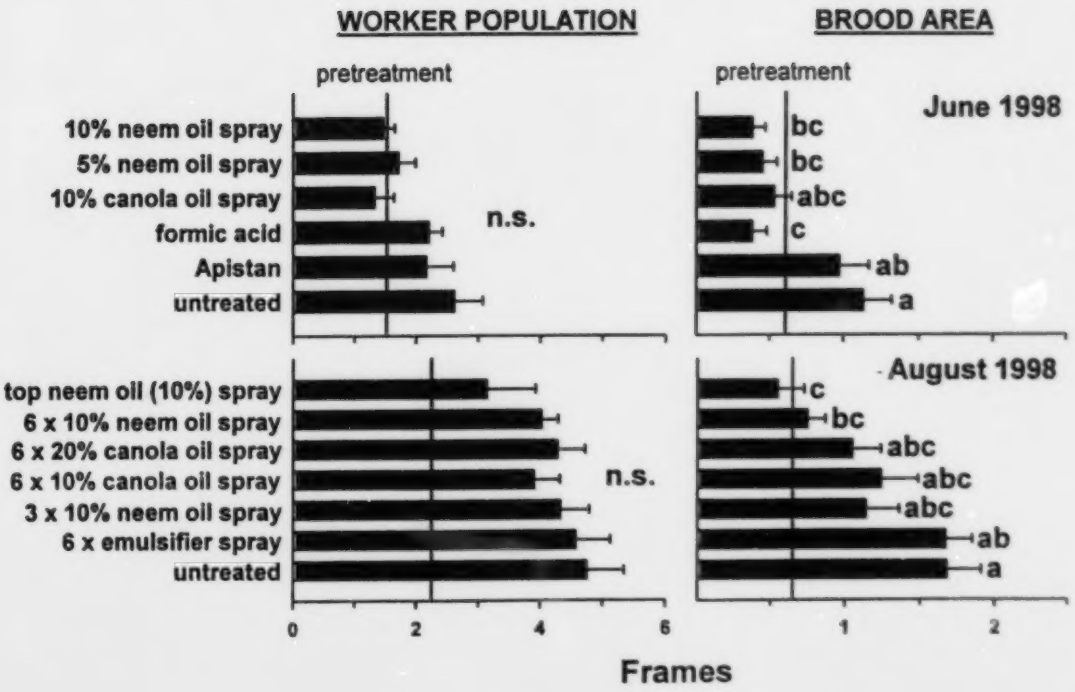
Figure 6. Mean (± SE) tracheal mite host transfer among bees treated with neem

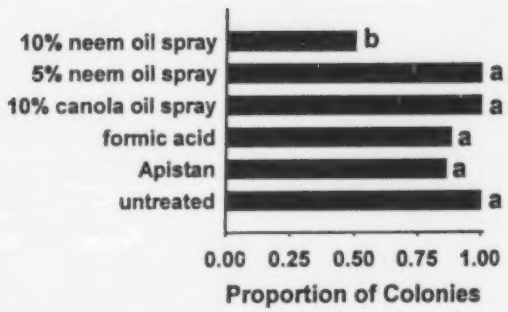
and canola oil or formic acid. Treatments followed by the same letter indicate no significant difference in host transfer (transformed) (Tukey-Kramer HSD, $P = 0.05$).

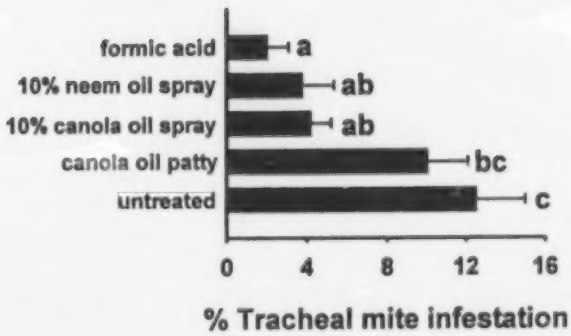

formic acid
10% neem oil spray
1% neem oil spray
canola oil patty
neem oil patty
9ml/L azasyrup
3ml/L azasyrup
untreated
a
ab
b
b
b
b
b
b
August 1997 (A)
Apistan
10% neem oil spray
formic acid
17ml/L azasyrup
debitterized neem oil patty
untreated
a
b
b
c
c
c
October 1997 (B)
Apistan
formic acid
10% neem oil spray
5% neem oil spray
10% canola oil spray
untreated
a
b
bc
bc
c
d
June 1998 (C)
top neem oil (10%) spray
6 x 10% neem oil spray
20% canola oil spray
10% canola oil spray
3 x 10% neem oil spray
emulsifier only spray
untreated
a
a
a
ab
abc
bc
c
August 1998 (D)
0
20
40
60
80
100
% Varroa mortality


Consumption (g)
crude neem oil
August 1997
b
a
100
80
60
40
20
0
debitterized neem oil
October 1997
a
a
neem oil
canola oil


Syrup consumed (kg)
0
1
2
3
4
5
a
b
neem aza
untreated


WORKER POPULATION
BROOD AREA
pretreatment
pretreatment
June 1998
10% neem oil spray
5% neem oil spray
10% canola oil spray
formic acid
Apistan
untreated
n.s.
bc
bc
abc
c
ab
a
August 1998
top neem oil (10%) spray
6 x 10% neem oil spray
6 x 20% canola oil spray
6 x 10% canola oil spray
3 x 10% neem oil spray
6 x emulsifier spray
untreated
n.s.
c
bc
abc
abc
abc
ab
a
0
2
4
6
1
2
Frames


10% neem oil spray
5% neem oil spray
10% canola oil spray
formic acid
Apistan
untreated
b
a
a
a
a
a
0.00 0.25 0.50 0.75 1.00
Proportion of Colonies


formic acid
10% neem oil spray
10% canola oil spray
canola oil patty
untreated
a
ab
ab
bc
c
0
4
8
12
16
% Tracheal mite infestation

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APICULTURE AND SOCIAL INSECTS

Comparative Laboratory Toxicity of Neem Pesticides to Honey Bees (Hymenoptera: Apidae), Their Mite Parasites *Varroa jacobsoni* (Acari: Varroidae) and *Acarapis woodi* (Acari: Tarsonemidae), and Brood Pathogens *Paenibacillus* Larvae and *Ascophaera apis*

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J. Econ. Entomol. 93(2): 000–000 (2000)

ABSTRACT Laboratory bioassays were conducted to evaluate neem oil and neem extract for the management of key honey bee (*Apis mellifera* L.) pests. Neem pesticides inhibited the growth of *Paenibacillus larvae* (Ash, Priest & Collins) in vitro but had no effect on the growth of *Ascophaera apis* (Olive & Spiltoir). Azadirachtin-rich extract (neem-aza) was 10 times more potent than crude neem oil (neem oil) against *P. larvae* suggesting that azadirachtin is a main antibiotic component in neem. Neem-aza, however, was ineffective at controlling the honey bee mite parasites *Varroa jacobsoni* (Ouduemans) and *Acarapis woodi* (Rennie). Honey bees also were deterred from feeding on sucrose syrup containing >0.01 mg/ml of neem-aza. However, neem oil applied topically to infested bees in the laboratory proved highly effective against both mite species. Approximately 50–90% *V. jacobsoni* mortality was observed 48 h after treatment with associated bee mortality lower than 10%. Although topically applied neem oil did not result in direct *A. woodi* mortality, it offered significant protection of bees from infestation by *A. woodi*. Other vegetable and petroleum-based oils also offered selective control of honey bee mites, suggesting neem oil has both a physical and a toxicological mode of action. Although oils are not as selective as the *V. jacobsoni* acaricide τ-fluvalinate, they nonetheless hold promise for the simultaneous management of several honey bee pests.

KEY WORDS *Varroa, Acarapis*, acaricide, bioassay, neem, oil, τ-fluvalinate

COLONIES OF THE honey bee, *Apis mellifera* L., are susceptible to a number of pests and diseases whose damage has serious negative economic implications for both the beekeeping industry and agriculture. These pest problems have been further aggravated by the recent introduction of two parasitic mites into North America, the *Varroa* [*Varroa jacobsoni* (Oudemans)] and tracheal [*Acarapis woodi* (Rennie)] mites. Together these two mites species have severely reduced the number of healthy colonies available for beekeeping (Matheson 1994, Kraus and Page 1995, Finley et al. 1997), a service valued at more than $10 billion annually in North America alone (Robinson et al. 1989, CAPA 1995). Beekeepers have become increasingly dependent on the use of pesticides to combat bee pests leading to several problems, including increased treatment and labor costs, toxicological hazards to beekeepers and bees (Marchetti et al. 1987, Peng et al. 1992, Birnie and Winston 1998), risks of contaminating hive products (Furgala 1962, Lehnert and Shimanuki 1981, acaricides reviewed in Wallner 1999), and vulnerability to the evolution of pesticide resistance caused by the limited number of control agents available (reviewed in Milani 1999).

The seed kernel oil of the Asian neem tree, *Azadirachta indica* A. Juss., may offer a solution to these problems. Neem oil extracts have considerable broad-spectrum toxicity against a number of agricultural arthropod pests and pathogens (Quarles 1994, Schmutterer 1995) and could control multiple species of honey bee mite parasites and diseases simultaneously, thereby reducing the number of chemicals used in bee hives. Neem pesticides also have low environmental persistence (Sundaram and Curry 1994), do not induce resistance readily in insects (Feng and Isman 1995), and are relatively nontoxic to mammals (Larson 1989, Jacobson 1995). Although honey bee larvae are susceptible to azadirachtin-enriched neem insecticides (Rembold et al. 1980, Naumann and Isman 1996), they are less susceptible than other insect species (reviewed in Mordue and Blackwell 1993), suggesting that neem may be effective in killing honey bee pests at concentrations safe to the resident bees. Moreover, honey bees may not be susceptible to the most promising acaricides contained in the relatively noninsecticidal, azadirachtin-poor, nonpolar fractions of neem oil (Mansour and Ascher 1983, Sanguanpong and Schmutterer 1992, Mansour et al. 1993). Thus, neem-

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based pesticides are excellent candidates to test for controlling honey bee pests.

The objective of this study was to assess the relative toxicity of crude neem oil and a commercially available neem insecticide to adult honey bee workers and four pests that have worldwide economic significance: *Varroa* and tracheal mites and two microbial pathogens, the bacterium *Paenibacillus larvae* (Ash, Priest & Collins) [formely *Bacillus larvae* (White)] that causes American foulbrood disease, and the fungus *Ascophaera apis* (Olive & Spiltoir) that causes chalkbrood. These pests are from groups of organisms that were susceptible to neem pesticides in preliminary qualitative studies (Bunsen 1992; Liu 1995a, 1995b; Williams et al. 1998). We screened the most effective neem pesticides and methods of exposure for all four pests by using laboratory bioassays.

Materials and Methods

Chemicals. Cold pressed neem seed kernel oil (neem oil) and unformulated azadirachtin-rich (10% azadirachtin wt:wt) neem seed insecticide (neem-aza) were gifts from Neem International Enterprises (Surrey, British Columbia). Additional batches of neem oil were compared in experiment 4 and were gifts from Thermo Trilogy (Columbia, MD), Trifolio-M GmbH (Lahnau, Germany), and Fortune Bio-Tech (Secunderabad, India). τ-Fluvalinate is a highly selective *Varroa* acaricide (Herbert et al. 1988). Technical grade and formulated τ-fluvalinate in a slow-release strip (Apistan) served as standards in *Varroa* bioassays (Wellmark International, Dallas, TX). Menthol vapor is a tracheal mite acaricide (Vecchi and Giordani 1968, Ellis and Baxendale 1997) and served as the standard for tracheal mite bioassays (Sigma, Oakville, ON). Citronellal, clove oil, and cinnamon oil have established minimum inhibitory concentrations for *P. larvae* in vitro (Calderone et al. 1994) and were used as standards in bioassays (Sigma). Canola seed oil (CountryPure, Lucerne Foods, Vancouver, BC), peanut oil (Planters, Hershey Canada, Mississauga, ON), grape seed oil (Borges, Jentash Marketing, Delta, BC), mineral oil (Sigma), and Stylete horticultural oil (JMS Flower Farms, Vero Beach, FL) were compared with neem oil in bioassays. Vegetable oils have acaricidal activity against tracheal mites (Smith et al. 1990, Calderone and Shimanuki 1995) and also were used as standards in tracheal mite bioassays. Neem-aza powder and menthol crystals were dissolved and diluted in EtOH, and τ-fluvalinate, citronellal, clove oil, cinnamon oil, neem oil, and vegetable and petroleum oils in hexane.

Antibiotic Bioassays. Growth inhibition of *A. apis* and *P. larvae* by neem pesticides was determined in vitro using microbiological medias and bioassays from Calderone et al. (1994). The gram-positive bacterium *Bacillus cereus* (Carolina Biological, Microkwik Culture) and the gram-negative bacterium *Escherichia coli* (Carolina Biological, Microkwik Culture) were included in the study to determine if antibiotic activity related to gram staining characteristics.

A stock culture of *A. apis* (ATCC 56293) mycelia was grown for 7 d before the start of the experiment on potato dextrose agar containing 0.4% yeast extract (PDAY) (Calderone et al. 1994) at 30°C. *P. larvae* (ATCC 25748), *B. cereus*, and *E. coli* inocula were cultivated in brain heart infusion broth with thiamine (BHIT) (Calderone et al. 1994) at 35°C until the density of colony forming units was approximately 3×10^7, 5×10^7, and 5×10^9 organisms per milliliter, respectively. Stock cultures served as inocula for the bioassays and bacterial cultures were vigorously agitated immediately before inoculation.

Bioassays were conducted to evaluate microbial growth on solid agar media containing varying concentrations of neem pesticide or antibiotic standard. Neem-aza was incorporated into PDAY and BHIT agar at concentrations of 0, 0.05, 0.09, 0.19, 0.38, 0.75, 1.5, 3, and 6 mg/ml of media. EtOH was used to dissolve and dilute neem-aza and remained constant at 6.4 μl/ml across all concentrations. Neem oil and antibiotic standards citronellal, clove oil, and cinnamon oil were incorporated directly into media. Neem oil concentrations were 0, 0.02, 0.04, 0.09, 0.17, 0.34, 0.68, 1.37, and 2.7 mg/ml and the standards were 0.04, 0.34, and 2.7 mg/ml. Standards were used for all experiments except for *A. apis* bioassays. All test substances were combined with molten media at 65°C and then immediately poured into petri dishes. Moderate agitation immediately before pouring plates ensured even distribution of test compounds through the agar matrix. Each concentration was replicated in three different dishes for *A. apis* and four dishes for the bacteria.

A single 4-mm disk of mycelia, obtained from the stock culture, served as the inocula for *A. apis* bioassays. Radial mycelia growth at 72 h was estimated as the sum of four perpendicular measurements from the edge of the inoculum disc to the outer growing edge of the mycelia. The minimum inhibitory concentration (MIC) of neem pesticides to bacteria was determined by inoculating plates with 100 μl of stock culture and visually inspecting for the absence or presence of growth at 48 h.

Bees and Mites. All bees and mites used in experiments came from honey bee colonies treated with the antibiotics oxytetracycline hydrochloride (OxyTet-25 - Medivet Pharmaceuticals, High River, AB) and bicyclohexylammonium fumagillin (Fumagilin B - Medivet Pharmaceuticals) for the control of microbial pathogens. Source colonies were not treated with acaricides for at least 1 mo before conducting experiments. The honey bees could not be classified into a specific race of *A. mellifera* because stocks of several different races have been imported to the region and not selected for racial characteristics. Two distinct haplotypes of *V. jacobsoni* exist in North American, one originating from the Russian Far East and the other from Japan/Thailand (Guzman et al. 1999). Genetic analysis of *V. jacobsoni* specimens obtain British Columbia suggest mites in our study area were Russian (Guzman et al. 1999).

Mite Bioassays. Four experiments were conducted to evaluate the toxicity of neem pesticides to honey

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Table 1. Treatments used to evaluate the toxicity of neem to *Varroa* adults and adult worker honey bees (experiment 1)

Treatment	Application method	Treatment rate[a]	Concn
Untreated	—	—	—
Untreated	Vapor	—	—
Solvent control (EtOH)	Oral	200 μl	10 μl/ml
Solvent control (EtOH)	Topical	2 μl	—
Neem oil	Vapor	5 ml	—
Neem aza	Oral	200 μl	0.43 mg/ml
Low neem aza	Topical	2 μl	4.3 mg/ml
High neem aza	Topical	2 μl	430 mg/ml
Low neem oil	Topical	2 μl	—
High neem oil	Topical	4 μl	—
Apistan	Topical	3-cm² strip	10% (wt:wt)

[a] Oral and vapor-treatment volume expressed as applied per cage and topical treatments as applied per bee. Apistan treatments expressed as exposed strip surface area per cage.

Table 2. Treatments used to evaluate the toxicity of neem to tracheal mite and adult worker honey bees (experiment 2)

Treatment	Application method	Treatment rate[a]	Concn
Untreated	—	—	—
Solvent control (EtOH)	Topical	2 μl	—
Neem oil	Vapor	5 ml	—
Neem aza	Oral	800 μl	0.43 mg/ml
Neem aza	Topical	2 μl	430 mg/ml
Neem oil	Topical	2 μl	—
Grape seed oil	Topical	2 μl	—
Menthol	Vapor	20 mg	—

[a] Oral and vapor treatment volume expressed as applied per cage and topical treatments as applied per bee.

bees and mites. Experiments were conducted on mite-infested adult workers held in cages. Bioassays compared the mortality of bees and mites receiving neem treatments with untreated groups or groups treated with acaricides selectively toxic to mites.

Three different cage designs were used in the experiments: mesh, plastic cup, and petri dish. Mesh cages were 300-ml cylinders with fine wire mesh walls and solid lids and bases made from 100 by 50-mm plastic petri dishes. Plastic cup cages were 250-ml plastic cups with screened ventilation holes and a solid plastic base. Petri dish cages were 60 by 30-mm polystyrene petri dishes.

Gravity-fed syrup feeders were filled with a 3 M sucrose solution and fitted through a hole punched in the lids of mesh and plastic cup cages. Syrup feeders were made from 10 by 75-mm disposable polypropylene snap-cap tubes (Fisher, Mississauga, ON) punched with a 22-gauge needle at the tapered end. Unless specified, syrup was fed ad libitum. Bees in petri dish cages were fed from a solid 3.5-g sucrose cube. Bees held in mesh and plastic cup cages were incubated at 30°C and 50% RH, whereas bees in petri dish cages were held at 30°C and 70% RH, which provided sufficient moisture for consumption of the solid sucrose.

Treatment and cage location in incubators was randomized for all experiments. Bees physically deformed by *Varroa* parasitism during the pupal stage were not used in experiments.

Experiment 1. Toxicity of Neem to Varroa and Bees. Worker bees were collected directly into mesh cages from brood frames of colonies heavily infested with *Varroa*. Approximately 20 bees and 8 adhering *Varroa* were put into each cage. The treatments assigned to the cages are described in Table 1. Bees and mites were exposed to neem oil vapor by sealing cages in 4-liter glass jars containing a 10-dram vial with 5 ml of neem oil. Untreated cages also were sealed within glass jars and served as a control for neem-oil-vapor-treated cages. Neem-aza was fed to the bees in sucrose syrup, and once the treatment was consumed, bees were fed untreated syrup ad libitum. Topical neem and solvent-only treatments were applied to the thorax of bees immediately before being placed in cages by using a micropipette. Apistan was applied to cages as a 1 by 3-cm strip placed on the cage base (acaricide standard). Each treatment was replicated eight times.

The number of dead *Varroa* and bees was tallied after visual inspection of the cage bottoms. Bee and mite mortality was assessed 48 h after treatment except for vapor treatments that were assessed after 7 d.

Experiment 2. Toxicity of Neem to Tracheal Mites and Bees—Effects on Tracheal Mite Host Location. Mesh cages were each filled with 70 worker bees collected directly from the upper lid of colonies where >80% of bees were infested with tracheal mites. Thirty newly eclosed workers also were added to each cage. Newly eclosed workers serve as host targets for dispersing mites (Gary et al. 1989, Smith et al. 1990) and were used to test if neem treatments disrupted tracheal mite host transfer. Newly eclosed workers were obtained from frames of sealed brood emerged in an incubator at 32°C for 18 h. An enamel paint mark (Testors, Weston, ON) on the tip of the abdomen distinguished these bees from infested bees already in the cage. The paint marks themselves do not influence mite host location (Smith et al. 1990).

The treatments assigned to the cages are described in Table 2. Treatment application methods were identical to those described for experiment 1, except all cages, not only those receiving vapor treatment, were sealed in 4-liter glass jars to permit direct comparison to the menthol vapor acaricide standard. Each treatment was replicated five times. Bee and tracheal mite mortality was assessed 7 d after treatment. Adult tracheal mite mortality among infested unmarked bees was determined by live dissection of the main prothoracic tracheal trunks (Eischen et al. 1987) and a total of five tracheal-mite-infested bees was assessed per cage. In addition, 10 marked host bees were killed in 70% EtOH and then scored for the presence or absence of mites.

Experiment 3. Toxicity of Long-term Exposure of Honey Bees, Varroa, and Tracheal Mites to Neem and Vegetable Oil. Approximately 40 bees were collected into plastic cup cages from a single colony with 50% worker tracheal mite infestation. Cages were transported immediately from the field and placed into an incubator. Five bees carrying *V. jacobsoni* were then

Varroa

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Table 3. Origin of neem oil products used in experiment 4

Identification	Manufacturer	Batch	Geographic location of seed source
Neem A	Fortune Bio-Tech[a]	1	India
Neem B	Fortune Bio-Tech	2	India
Neem C	Thermo Trilogy[b]	1	India
Neem D	Thermo Trilogy	2	India
Neem E	Trifolio-M[c]	1	India
Neem F	Trifolio-M	2	India
Neem G	Neem Int. Enterprise[d]	1	Australia

[a] 14 Ishaq Colony, 108 Bazar Road, Secunderabad-500 015 (A.P.) India.
[b] 9145 Guilford Road, Suite 100, Columbia, MD 21046.
[c] Sonnestr .22, D-35633 Lahnau, Germany.
[d] 5644- 132nd Street, Surrey, BC V3X 1N5. Neem G was used in experiments 1–3.

added to each cage. The *Varroa*-infested bees were obtained from frames of worker brood emerged during 24 h in an incubator.

Treatments were applied as a residual film on the base of each cage. Cages either remained untreated or were treated either with 45 or 90 μl of residual neem or canola oil. Oil treatments were reapplied at 48-h intervals for 10 d. Each treatment was replicated five times. The number of dead *Varroa* and bees was tallied after visual inspection of the cage bottoms every 24 h for a total of 12 d. Adult tracheal mite mortality among infested bees was determined at the end of the experiment by dissecting the trachea of live bees. At least five bees infested with tracheal mites were dissected per cage.

Experiment 4. Comparative Toxicity of Different Vegetable and Mineral Oils and Batches of Neem Oil to Varroa and Bees. A uniform film of test material was deposited on the base of petri dish cages by pipetting 1 ml of treatment dissolved and diluted in hexane. The hexane was completely evaporated from the dishes by exposure to forced airflow for 10 min and cages were then stored at −20°C for 5 d. Cages were kept at room temperature for 1 h before use in bioassays.

Sealed brood from colonies heavily infested with *Varroa* was allowed to emerge in an incubator for 24 h, after which time six newly eclosed bees carrying on average six mites were added to each cage.

Control treatments included cages that remained untreated and cages treated with hexane solvent alone. Experimental oil treatments were applied at rates of 6, 15, 37.5, and 75 μl per cage. Oils tested were canola, peanut, mineral, Stylete, and seven different batches of neem obtained from different geographic locations and manufacturers (Table 3). τ-Fluvalinate was applied at rates of 1, 10, 20, and 200 μg per cage. Each treatment × dose combination was replicated in six cages. The number of dead *Varroa* and bees was tallied after visual inspection of the cage bottoms at 24 and 72 h.

Palatability Bioassays. The palatability of syrup treated with neem-aza was determined by collecting ~15 bees into mesh cages and feeding them syrup containing either no treatment (control); neem-aza at 0.004, 0.009, 0.017, 0.034, 0.069, 0.138, 0.275, 0.55, and 1.1 mg/ml syrup; or EtOH (1.1 mg/ml, solvent control). Each treatment × concentration combination was replicated five times. The amount of syrup consumed per cage during a 48-h period was determined gravimetrically and converted to syrup consumed per bee by dividing the number of bees in each cage at the start of the experiment. A second experiment compared consumption from a second feeder containing untreated syrup, thus providing the bees a choice between treated and untreated syrup.

Data Analysis. Antibiotic Bioassays. The relationship between neem concentration and *A. apis* radial growth was modeled using least square means linear regression and the effect of dose on growth tested using analysis of variance (ANOVA) (SAS Institute 1997), with concentration transformed using a base-10 logarithm function (Sokal and Rolf 1981). Radial growth of *A. apis* on media containing EtOH was compared with growth on untreated media using a two-tailed Student *t*-test (SAS Institute 1997).

Mite Bioassays. Mortality of *Varroa* and bees was determined as the number dead divided by the total per cage. Mortality of adult tracheal mites was calculated as the mean mortality of mites per bee per cage. For experiments 1, 2, and 3, the hypothesis that treatment had no effect on either bee or mite mortality was tested using a one-way ANOVA (SAS Institute 1997) using root arcsine-transformed mortality as the dependent variable (Sokal and Rolf 1981). Treatment differences for transformed mean mortality were compared using Tukey–Kramer honest significant differences test (SAS Institute 1997).

The relationship between concentration and mortality for bees and *Varroa* in experiment 4 was modeled using logistic regression (SAS Institute 1997). Bee LC_{30}, *Varroa* LC_{70}, and inverse 95% fiducial limits (FL) were estimated for each treatment. *Varroa* LC_{70} was selected because acaricide treatments are considered ineffective unless >70% of mites are killed in field situations (Koeniger and Fuchs 1989). Conversely, bee LC_{30} was used because acaricides must have minimal effects against bees, and 30% bee mortality represented the most accurate estimate of low bee mortality. Selective toxicity against *Varroa* was considered significant when the 95% FL for *Varroa* LC_{70} and bee LC_{30} failed to overlap. A selectivity index was calculated for each treatment by dividing bee LC_{30} by *Varroa* LC_{70} to determine which treatments had the greatest potential as acaricides.

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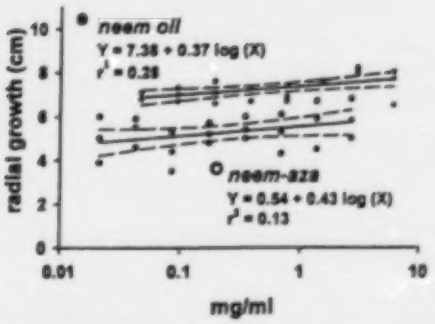

Fig. 1. Radial growth of *A. apis* 72 h after inoculation on artificial media treated with either neem oil or neem-aza (±95% CL). There was no significant relationship between growth and treatment concentration. Each concentration was replicated three times.

Palatability Bioassays. The relationship between concentration of neem-aza and syrup consumption was modeled using least square means linear regression (SAS Institute 1997). The effect of concentration on consumption was tested statistically using an ANOVA. The hypothesis that EtOH alone reduced syrup consumption was tested using a two-tailed Student *t*-test (SAS Institute 1997).

Results

Antibiotic Bioassays. *A. apis* radial mycelia growth was not significantly affected by media concentration of either neem oil ($F = 3.10$; df = 1, 22; $P = 0.092$) or neem-aza ($F = 3.42$; df = 1, 22; $P = 0.078$) (Fig. 1). The concentration of EtOH used to dilute neem-aza, however, resulted in a 20% reduction in radial mycelia growth when compared with cultures grown on untreated media ($t = 6.48$, df = 4, $P = 0.003$). The inhibitory effect of EtOH explains the slower growth rate of *A. apis* on neem-aza media compared with neem oil media, which lacked EtOH (Fig. 1).

Paenibacillus larvae growth in vitro was completely inhibited by both neem-aza and neem oil. However, neem-aza was ~10 times more potent (Fig. 2). Minimum inhibitory concentrations of botanical antibiotic standards for *P. larvae* were similar to Calderone et al. (1994) (0.01–0.8 mg/ml) and were generally lower than that of either neem-aza (0.3 mg/ml) or neem oil (3.0 mg/ml). Neither *B. cereus* nor *E. coli* was inhibited by the concentrations of neem oil or neem-aza used in this study. *B. cereus* and *E. coli* also tended to be less sensitive than *P. larvae* to the antibiotic standards.

Mite Bioassays. *Experiment 1. Toxicity of Neem to Varroa and Bees.* Neem oil applied topically at a rate of 4 μl per bee resulted in the mortality of 45% of the Varroa per cage, higher than the mortality observed in untreated cages (Fig. 3A). Treatment with the known anti-*Varroa* acaricide Apistan, however, resulted in almost complete mite mortality. In contrast, treatment with neem-aza, applied topically or fed to bees, did not result in significant mite mortality. Treatment with neem oil vapor also did not result in significant mite mortality ($t = 0.92$, df = 14, $P = 0.372$) (Fig. 3B). No differences in bee mortality were observed among oral or contact treatments at 48 h ($F = 0.60$; df = 6, 49; $P = 0.605$) or between vapor treatments at 7 d ($t = 0.64$, df = 14, $P = 0.534$) with mortality per cage averaging 4.9 ± 3.0 and 6.3 ± 1.1%, respectively.

Experiment 2. Toxicity of Neem to Tracheal Mites and Bees—Effects on Tracheal Mite Host Location. No neem treatments were as effective at killing adult tracheal mites as menthol, a standard acaricide (Fig. 4A). None of the treatments, including menthol, affected the number of larval mites (4.35 ± 2.17 larvae per bee) ($F = 1.17$; df = 8, 34; $P = 0.342$) or eggs (5.47 ± 2.64 eggs per bee) ($F = 1.12$; df = 8,34; $P = 0.306$) found in trachease. Topical application of neem oil, however,

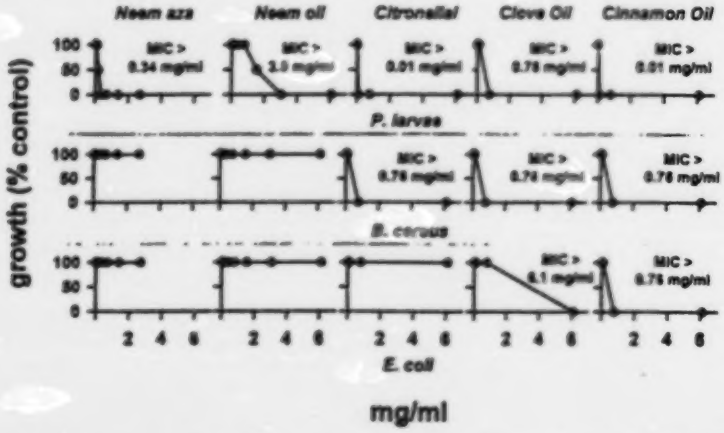

Fig. 2. Minimum inhibitory concentration (MIC) of neem and antibiotic standards for *P. larvae*, *B. cereus*, and *E. coli*. The MICs were determined 48 h after inoculation. Each concentration was replicated four times.

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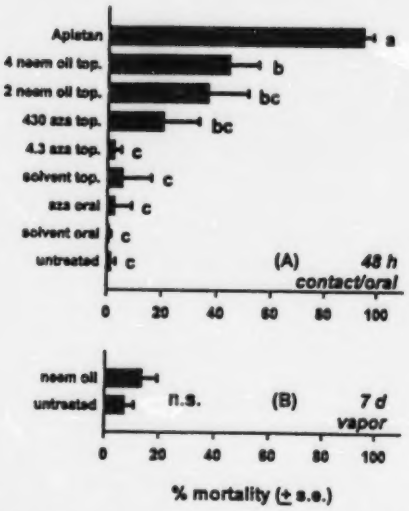

Fig. 3. *Varroa* mortality of infested bees treated with neem-oil or neem-aza. Treatments were administered to bees (A) topically on the thorax or in sucrose syrup feed (oral), or (B) as a vapor. Apistan, the positive acaricide control was applied as a contact strip. Oral and contact treatments were assessed 48 h after treatment and vapor treatments after 7 d. Differences in mite mortality existed only among oral and topical treatments ($F = 15.54$; df = 6, 49; $P < 0.001$). Treatments followed by the same letter indicate no significant difference in transformed *Varroa* mortality (Tukey–Kramer honestly significant difference [HSD], $P = 0.05$) ($N = 8$).

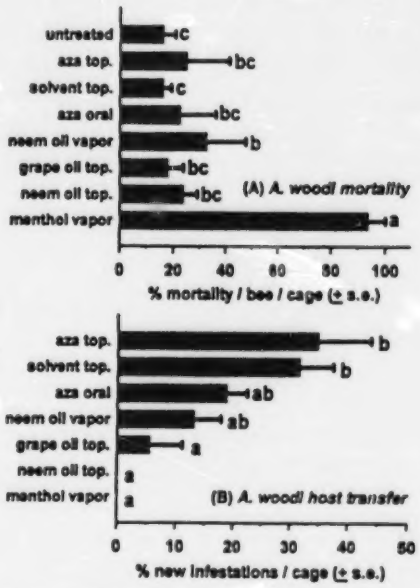

Fig. 4. Tracheal mite (A) mortality and (B) host transfer among bees treated with neem-oil or neem-aza. Treatments were administered to bees topically on the thorax, in sucrose syrup feed (oral), or as a vapor. Menthol vapors and grape seed oil acted as the positive acaricide controls. Cages were assessed for mite mortality and transfer 7 d after treatment. Treatment differences existed for both mite mortality ($F = 17.54$; df = 7, 38; $P < 0.001$) and mite host transfer ($F = 7.80$; df = 6, 24; $P < 0.001$). Treatments followed by the same letter indicate no significant difference in root arcsine transformed tracheal mite mortality or infestation rate (Tukey–Kramer HSD, $P = 0.05$) ($N = 5$).

resulted in low rates of tracheal mite host transfer comparable to groups treated with the acaricide standards menthol and grape seed oil (Fig. 4B). Remarkably, no marked susceptible bee treated with neem oil was infested with tracheal mites. Adult bee mortality was comparable among treatments ($F = 1.98$; df = 8, 34; $P = 0.079$) and averaged 2.5 ± 1.7% per cage.

Experiment 3. Toxicity of Long-Term Exposure of Honey Bees, Varroa, and Tracheal Mites to Neem and Vegetable Oil. Treating bees with higher doses of neem resulted in approximately ~95% *Varroa* mortality (Fig. 5A). Comparable *Varroa* mortality was achieved using canola oil. Both oil treatments killed most *Varroa* within 48 h of treatment (Fig. 6). Treatment with 90 μl of canola oil, however, resulted in significant bee mortality (Fig. 5B). Although bee mortality in cages treated with 90 μl of neem oil was not statistically significant, it was approximately twice that of the untreated group.

Oil treatments had no effect on tracheal mite mortality ($F = 0.25$; df = 4, 19; $P = 0.904$), despite the higher doses and treatment duration used. Tracheal mite mortality per bee per cage averaged 29.4 ± 7.1% across all treatments.

Experiment 4. Comparative Toxicity of Different Vegetable and Mineral Oils and Batches of Neem Oil. τ-Fluvalinate proved 20–100 times more selective at killing *Varroa* than oils (Table 4; Fig. 7). Longer exposure time (72 h) appeared to increase the selectivity of most treatments. All oil treatments were more selective against *Varroa* than bees and by 72-h selectivity indices ranged from 1.5 to 10. Some treatments had *Varroa* LD_{70} and bee LD_{30} confidence limits (CL) that overlapped, suggesting nonsignificant selectivity for those treatments. The selectivity index varied among neem oil products from different manufacturers and among batches from the same manufacturer. Although petroleum-based mineral and Stylete oil differed in selectivity, vegetable-based peanut and canola oils had similar low selectivity indices and high *Varroa* LD_{70} and bee LD_{30} values compared with other treatments. Based on the selectivity index at 72 h the treatment with the greatest potential for the control of *Varroa* was neem oil batch F.

Palatibility Bioassays. Honey bee syrup consumption was reduced with increasing concentration of neem-aza (Fig. 8), whether bees were given a choice between treated or untreated syrup sources ($F = 84.60$; df = 1, 48; $P < 0.001$) or not ($F = 107.37$; df = 1, 48; $P < 0.001$).

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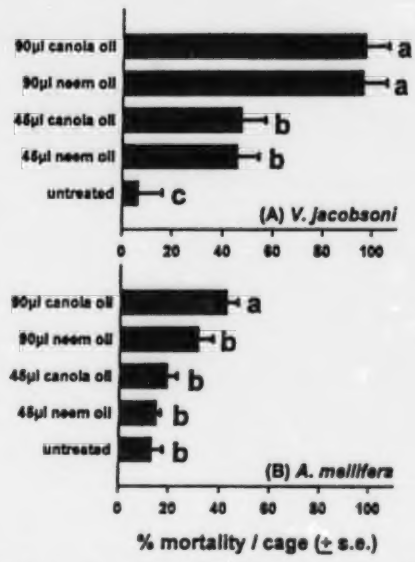

Fig. 5. Mortality of (A) *Varroa* and (B) bees treated with neem or canola seed oil. Treatments were administered as a residual film on the base of each cage and were reapplied at 48 h intervals for 10 d. Cages were assessed for mite and bee mortality 12 d after the first treatment. Treatment differences existed for both mite mortality ($F = 22.35$; df = 4, 19; $P < 0.001$) and bee mortality ($F = 5.04$, df = 4, 18, $P = 0.007$). Treatments followed by the same letter indicate no significant difference in root arcsine transformed *Varroa* or bee mortality (Tukey–Kramer HSD, $P = 0.05$) ($N = 5$).

Discussion

These laboratory bioassays demonstrate that neem pesticides have no effect on the growth of *A. apis*, inhibit *B. larvae*, and control the mites *V. jacobsoni* and *A. woodi*. Neem-aza pesticides proved more potent than neem oil at controlling *P. larvae* but only neem oil proved effective against honey bee mites. Neem oil killed *Varroa* on contact but not tracheal mites, although topically applied neem oil protected susceptible uninfested bees from tracheal mite infestation. Other vegetable and petroleum-based oils also offered selective control of honey bee mites. Collectively, the results suggest neem and other oils hold promise for the simultaneous management of several honey bee pests and diseases.

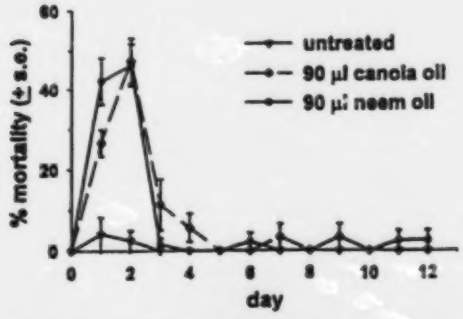

Fig. 6. Daily *Varroa* mortality after treatment with neem or canola oil at 48-h intervals for 10 d ($N = 5$).

Contrary to earlier findings (Liu 1995a), neem pesticides did not inhibit the growth of *A. apis* in vitro. Unformulated neem-aza and neem oil was used in the current study, whereas Liu (1995a) tested a formulated product. Materials used to formulate neem pesticides include solvents, UV screens, and antioxidants (Quarles 1994), which may themselves inhibit *A. apis* growth. For example, we observed that 6.4 µl/ml of EtOH resulted in substantial *A. apis* growth inhibition.

The minimum inhibitory concentration of unformulated neem-aza to *P. larvae* was ~10 times that observed by Williams et al. (1998) for pure azadirachtin. The neem-aza tested, however, was only 10% azadirachtin, suggesting *P. larvae* antibiotic activity depends on the concentration of azadirachtin. Further evidence for this hypothesis is that azadirachtin-poor neem oil was 10-fold less potent than neem-aza (Fig. 2).

Paenibacillus larvae spores are resistant to most forms of chemotherapy. Therefore, prevention of infection requires that sufficient antibiotic be present to inhibit the vulnerable vegetative growth in the gut and hemolymph of young larval bees (Bailey and Ball 1991). The ability of neem to prevent infections at doses safe to bees is a concern because the LD_{50} for a first-instar worker to azadirachtin is 37 µg/g (Naumann and Isman 1996), and we found that concentrations >30 µg/ml are required to inhibit growth. Thus, larval bees would likely die at much lower dose than needed for (*Paenibacillus* larvae) control. An in vivo bioassay comparing the dose of larval mortality with the dose needed to prevent infection (Peng et al. 1992) could test this hypothesis. Other botanical pesticides may offer an opportunity to control *P. larvae* because clove and cinnamon oils were more potent than neem-aza, but the toxicities of these putative botanical antibiotics to honey bee larva are unknown.

Neem-aza was ineffective at controlling honey bee mites in the laboratory, contrary to other published reports (Bunsen 1992, Liu 1995b). This result is not surprising because although azadirachtin is highly toxic to insects (Mordue and Blackwell 1993) it generally is not toxic to Acari (Mansour and Ascher 1983, Lindsay and Kaufman 1988, Sanguanpong and Schmutterer 1992, Mansour et al. 1993, Spollen and Isman 1996). The lack of neem-aza activity observed also may have resulted from insufficient absorption of azadirachtin into mites, because azadirachtin is poorly absorbed through arthropod cuticule (Paranagama et al. 1993) and little chemical may have entered mites by contact treatments. Because bees were strongly deterred from feeding on syrup treated with neem-aza, mites also would not have encountered systemic azadirachtin while feeding on honey bee hemolymph. The ability of azadirachtin to inhibit feeding is common among insects (Mordue and Blackwell 1993) and

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Table 4. Comparative toxicity of τ-fluvalinate, neem, and other oils to *Varroa* and adult worker honey bees

Treatment	Time, h	*Varroa*			Honey Bee			
		n	Slope ± SE	LC_{70} (95% FL) mg/cage	χ^2	*n*	Slope ± SE	LC_{30} (95% FL) mg/cage	χ^2
τ-fluvalinate	24	195	0.24 ± 0.04	7.46 (10.21–5.64)	13.5	197	0.01 ± 0.01	284 (731–209)[a]	24.1
	72		0.29 ± 0.07	3.09 (5.36–1.60)	0.02		0.00 ± 0.00	653[a,b]	58.9
Neem A	24	169	0.35 ± 0.08	6.24 (9.94–4.56)	29.8	179	1.08 ± 10.0	16.4[b]	0.02
	72		0.93 ± 1.83	2.14 (3.14–1.59)	17.6		0.21 ± 0.03	15.0 (17.7–12.2)	53.1
Neem B	24	192	0.25 ± 0.04	7.87 (11.1–5.96)	22.5	178	0.35 ± 0.08	13.1 (15.4–10.6)	44.9
	72		0.40 ± 0.11	3.91 (7.12–2.70)	8.49		0.22 ± 0.03	6.43 (8.55–4.20)	49.7
Neem C	24	186	1.54 ± 0.40	1.08 (2.00–0.70)	8.94	179	0.23 ± 0.03	7.72 (9.86–5.45)	51.9
	72		0.35 ± 0.06	0.73 (1.67–0.39)	0.65		0.35 ± 0.06	3.74 (5.43–2.27)	48.1
Neem D	24	229	0.54 ± 0.13	3.86 (6.62–2.78)	28.6	211	0.34 ± 0.04	6.44 (8.26–4.78)	63.7
	72		1.36 ± 0.29	1.33 (2.16–0.94)	19.6		0.47 ± 0.11	2.99 (4.68–1.98)	56.8
Neem E	24	199	0.50 ± 0.12	4.45 (7.52–3.22)	23.3	178	0.35 ± 0.08	14.6 (16.6–12.4)	24.4
	72		1.46 ± 0.24	1.70 (2.36–1.29)	27.7		0.27 ± 0.04	8.40 (10.4–6.24)	48.7
Neem F	24	190	0.56 ± 0.14	3.59 (6.09–2.61)	19.4	177	0.32 ± 0.05	9.85 (11.75–7.6)	38.5
	72		3.40 ± 2.38	0.61 (3.10–0.41)	15.0		0.27 ± 0.04	6.34 (8.29–4.36)	50.8
Neem G	24	174	0.34 ± 0.07	6.68 (10.4–4.94)	31.1	173	0.30 ± 0.05	17.9 (20.6–15.2)	36.8
	72		1.30 ± 0.22	1.90 (2.61–1.45)	25.6		0.35 ± 0.08	12.4 (15.0–9.71)	53.2
Mineral Oil	24	187	0.27 ± 0.06	5.93 (9.41–4.23)	11.9	180	0.51 ± 0.09	9.19 (11.1–6.45)	21.2
	72		3.40 ± 1.66	0.44 (6.32–0.26)	3.24		0.47 ± 0.10	4.36 (6.40–3.01)	51.0
Stylete Oil	24	192	0.85 ± 0.16	2.83 (3.80–2.25)	22.9	177	1.28 ± 11.5	12.9[b]	0.01
	72		0.97 ± 0.19	1.49 (2.25–1.02)	5.20		0.39 ± 0.06	5.37 (7.25–3.75)	51.8
Peanut Oil	24	146	0.22 ± 0.04	11.4 (15.2–9.00)	26.8	138	0.30 ± 0.06	21.2 (23.9–17.2)	24.8
	72		0.89 ± 0.21	2.90 (4.08–2.26)	11.8		0.24 ± 0.04	11.08 (13.6–8.24)	37.0
Canola Oil	24	165	0.23 ± 0.03	14.1 (17.5–11.8)	51.6	177	0.91 ± 7.28	28.4[b]	30.5
	72		0.93 ± 0.18	3.28 (4.41–2.65)	0.01		0.19 ± 0.03	15.5 (18.3–12.5)	54.3

Lethal dose and 95% FL were estimated using logistic regression (SAS Institute 1997).
[a] Less than 25% mortality observed at the highest concentration tested (200 μg/cage).
[b] Fiducial limits could not be calculated because of heterogeneous data.

has been described previously for foraging honey bees (Naumann et al. 1994).

Neem oil was effective at selectively killing *Varroa* and preventing the spread of tracheal mites. Although acaricidal properties of neem oil are known (Mansour and Ascher 1983, Sanguanpong and Schmutterer 1992, Mansour et al. 1993), its activity has been suggested to be unique and not generic to a wider subset of vegetable and petroleum-based oils. However, horticultural and veterinary oils are widely used as agents to control mite pests (Smith and Pearce 1948, Fiori et al. 1963, Guiamaraes and Tucci 1992, Agnello et al. 1994, Herron et al. 1996). Our results demonstrate that the acaricidal activity of neem oil is not a unique property but is shared by other oils.

Vegetable and petroleum-based oils offered comparable acaricide activity to neem oil in our study. Although vegetable oils are known to mechanically disrupt tracheal mite host location (Smith et al. 1990, Sammataro and Needham 1996), they have only recently been demonstrated to disrupt or kill *Varroa* (Le Conte et al. 1998). The acaricidal effects of neem oil against *Varroa* and tracheal mites are, therefore, partially the result of its physical properties. Neem oil

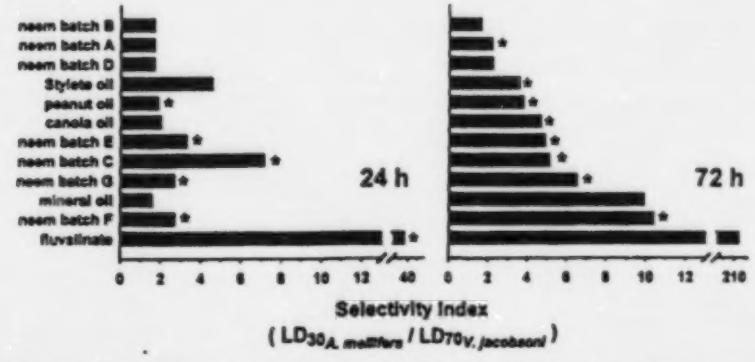

Fig. 7. Bee LC_{30} and *Varroa* LC_{70} estimates and calculated selectivity indices (bee LC_{30} / *Varroa* LC_{70}) for seven neem oil products, two vegetable oils, and two petroleum-based oils. τ-Fluvalinate acted as a positive acaricide control. Estimates were made after 24 and 72 h of exposure to a residual film of treatment on the surface of each cage. Indices followed by an asterisk have nonoverlapping 95% fiducial limits for bee LC_{30} and *Varroa* LC_{70}. Each concentration was replicated six times.

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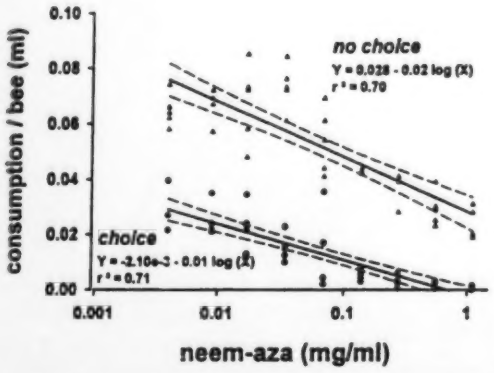

Fig. 8. Consumption of syrup treated with increasing amounts of neem-aza (±95% CL). Cages of bees were either given a choice between untreated and aza-treated syrup or given no choice. There was a significant negative relationship between syrup consumption and concentration of neem-aza both when bees were given a choice or not. Each concentration was replicated five times.

likely contains unique acaricides because some batches of neem oil were marginally more toxic to mites and safer for bees than other oils. Nonetheless, vegetable and mineral oils should also be investigated as possible colony treatments for honey bee mites.

Development of neem, vegetable and mineral oils into mite control products requires consideration of key issues identified by our research. Unlike τ-fluvalinate, oils possess a very narrow margin of toxicity between *Varroa* and bee mortality. Although τ-fluvalinate is ideally suited for *Varroa* management, widespread resistance (reviewed in Milani 1999) requires the development of alternatives, and to date most of these alternatives are less selective. Alternative acaricides currently used to manage honey bee mites, including thymol, menthol and formic acid, exhibit similar low selectivity as vegetable or mineral oils (Calderone et al. 1991, Ellis and Baxendale 1997, Imdorf et al. 1999, Lindberg et al. 2000). To achieve high levels of mite control without corresponding bee mortality requires oil treatment dosage to be applied with great precision. Also, our studies suggest that oils should be present for 48 h to be maximally effective, and applied topically rather than fed. Consequently, formulations of effective oil products require a continuous multiday uniform release.

An additional challenge is the batch variation in neem oil acaricide activity we observed, which could make standardization of treatments difficult. Isman et al. (1990) observed considerable variation in the insecticide and antifeedant activity of different neem oil batches, which correlated with azadirachtin content. Although the source of variation we observed is not caused by azadirachtin, neem oil contains many other potentially bioactive constituents that vary among crude extracts (Schmutterer 1995). Efforts to determine the source of laboratory variation and whether it translates into variation in field-mite control are critical.

Our methods allowed for rapid evaluation of many putative acaricides and application methods. Although a very selective acaricide (τ-fluvalinate) was confirmed using our methods, the ability to predict useful but less selective compounds requires more study. Nonetheless, our methods overcame drawbacks encountered by previous laboratory studies. Use of mites naturally infesting bees created test conditions more representative of the field compared with methods using isolated mites (Hoppe and Ritter 1989, Milani 1996, Elzen et al. 1998, Sammataro et al. 1998). Applying a residual film of test compound offers an alternative to treating individual insects (Eischen et al. 1987, Herbert et al. 1988, Calderone et al. 1991), which is time consuming and involves invasive manipulations such as anesthetization.

Varroa reproduction and development occurs on pupal honey bees, where mites are protected from acaricides by the wax cell capping. Acaricides have been evaluated against *Varroa* during pupal parasitism (Yoshida and Fuchs 1989, Bunsen 1992); however, none are effective. Although our bioassay examined the toxicity of neem to *Varroa* exclusively during its phoretic stage on honey bee adults, it is possible that neem also has an impact on the mite on pupae. Nonpolar fractions of neem oil reduce oviposition and disrupt nymphal development of spider mites of the genus *Tetranychus* (Mansour and Ascher 1983, Sanguanpong and Schmutterer 1992). Consequently, neem oil may not only control *Varroa* by killing phoretic adults but also by disrupting mite reproduction and development within cells.

The future direction of this work will be to evaluate neem and other oils in field settings. Studies are underway at Simon Fraser University to formulate and field test oils for the simultaneous control of *Varroa* and tracheal mites in operational bee colonies.

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Laboratory Evaluation of Miticides to Control *Varroa jacobsoni* (Acari: Varroidae), a Honey Bee (Hymenoptera: Apidae) Parasite

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ABSTRACT A laboratory bioassay was developed to evaluate miticides to control *Varroa jacobsoni* (Oudemans), an important parasite of the honeybee, *Apis mellifera* L. Bees and mites were exposed to applications of essential oil constituents in petri dishes (60 by 20 mm). The registered mite control agents tau-fluvalinate (Apistan) and formic acid also were evaluated as positive controls. Treatments that caused high mite mortality (>70%) at doses that produced low bee mortality (<30%) were considered mite selective. The six most selective of the 22 treatments tested (clove oil, benzyl acetate, thymol, carvacrol, methyl salicylate, and Magic3) were further evaluated to estimate LD_{50} values and selectivity ratios (*A. mellifera* LD_{50}/ *V. jacobsoni* LD_{50}) at 24, 43, and 67 h after exposure. Tau-fluvalinate was the most selective treatment, but thymol, clove oil, Magic3, and methyl salicylate demonstrated selectivity equal to or greater than formic acid. The effect of mode of application (complete exposure versus vapor only) on bee and mite mortality was assessed for thymol, clove oil, and Magic3 by using a 2-chambered dish design. Estimated *V. jacobsoni* LD_{50} values were significantly lower for complete exposure applications of thymol and Magic3, suggesting that both vapor and topical exposure influenced mite mortality, whereas estimated values for clove oil suggested that topical exposure had little or no influence on mite mortality. These results indicate that essential oil constituents alone may not be selective enough to control *Varroa* under all conditions, but could be a useful component of an integrated pest management approach to parasitic mite management in honey bee colonies.

KEY WORDS *Apis mellifera*, *Varroa jacobsoni*, bioassay, essential oils, tau-fluvalinate

Varroa jacobsoni (OUDEMANS) is a parasitic mite of the honey bee, *Apis mellifera* L., that is having a serious impact on managed and feral honey bee populations around the world. Infestations of this introduced mite in North America have reached epidemic proportions, with annual losses of untreated colonies soaring as high as 50–80% in some areas of the United States (Kraus and Page 1995, Finley et al. 1996). Beekeepers must purchase replacement colonies as well as expensive miticides to control and prevent infestations. These increased costs ultimately will be passed on to consumers and to those farmers that depend on rented honey bee colonies for crop pollination.

Varroa control requires that treatments be mite-selective (i.e., fatal to mites at doses that are harmless to bees), and leave no or minimal residues in honey and wax. Tau-fluvalinate, formulated and sold as Apistan, is the major *Varroa* control agent in the United States and Canada. Although tau-fluvalinate has been very effective at controlling mites (Ferrer-Dufol et al. 1991), it is expensive and leaves chemical residues in wax and honey that accumulate over time (Wallner 1995). Additionally, mites throughout Europe and North America are evolving resistance (Milani 1995, Elzen et al. 1998, Trouiller 1998). Other synthetic *Varroa* treatments such as flumethrin, coumaphos, and bromopropylate are labor-intensive, leave toxic residues (Lodesani et al. 1992, Wallner 1995), and are difficult to register in North America, although coumaphos currently is being used under emergency approval in the United States (Sanford et al. 1999). Formic acid, also registered for mite control in Canada and more recently in the United States, does not leave residues but requires multiple applications, poses a safety threat to the beekeeper, and is temperature-dependent in its efficacy (Hoppe et al. 1989, Imdorf et al. 1996). Thus, there is an urgent need for alternative treatments that are cost-effective, use an alternative mode of action or target site than tau-fluvalinate, and do not leave toxic residues in wax or honey.

Botanical products such as essential oil constituents and organic acids have demonstrated potential for mite control. They are inexpensive to produce and many such products have been exempted from the costly registration process in the United States and Canada because of their low mammalian toxicity (Quarles 1996). Residues from organic acid and essential oil treatments pose little toxicological concern to humans (Imdorf et al. 1995a, 1996; Bogdanov et al. 1998, 1999), although the residues of some essential oil constituents alter the taste of honey at high enough concentrations (Bogdanov et al. 1999).

Thymol, clove oil, menthol, methyl salicylate (wintergreen), and camphor have exhibited mite-selective properties in laboratory studies (Hoppe and Ritter 1989, Kraus et al. 1994, Imdorf et al. 1995b) and field

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experiments (Hoppe et al. 1989, Chiesa 1991, Calderone and Spivak 1995, Imdorf et al. 1996). Apilife-VAR (a blend of 76% thymol, 16.4% eucalyptol, 3.8% camphor, and 3.8% menthol) and Thymovar, another thymol-based product, are currently registered *Varroa* control treatments in Switzerland (Imdorf et al. 1995a, Andermatt 1999). Other organic acids and essential oil constituents have been found to be varroacidal (Colin et al. 1990, Imdorf et al. 1996, Sammataro et al. 1998) or effective against other agricultural mite pests (Larson and Berry 1984, Mansour et al. 1986, Calderone et al. 1991, Carrol 1994, Ellis 1997, Isman 1999).

There are three major criticisms of using plant-derived treatments for *Varroa* control, as follows: (1) applications are labor-intensive, requiring multiple treatments and monitoring (Imdorf et al. 1996); (2) their efficacy is variable (Pinzauti et al. 1988, Colin 1990, Calderone and Spivak 1995, Calderone et al. 1997), possibly as a result of the amount of brood present in the colony, the severity of the infestation, or the ambient temperature during treatment application; and (3) they have a narrow range of mite-selective doses (Kraus et al. 1994). Research is needed to elucidate their selectivity and search for application methods that are more selective, less variable, and easier to apply if these treatments are to realize their potential for *Varroa* control. The majority of research to date has focused on evaluating treatments at the colony level by using a limited range of doses and application methods (Pinzauti et al. 1988, Colin 1990, Chiesa 1991, Calderone and Spivak 1995, Sammataro et al. 1998). Laboratory bioassay experiments have focused solely on mite mortality, or have been limited in their choice of doses, application methods, and use of appropriate statistical analysis (Hoppe and Ritter 1989, Colin et al. 1990, Kraus et al. 1994, Imdorf et al. 1995b, Milani 1995, Sammataro et al. 1998).

The objective of this study was to develop an inexpensive and rapid cage-bioassay system to examine the acute toxicity of essential oil constituents to *A. mellifera* and *V. jacobsoni*. We developed a system that tested bees and mites simultaneously and allowed us to investigate the effects of dose, exposure time, and mode of application. We screened 22 essential oil constituents for their mite selectivity, then we tested the most promising treatments to estimate mite and bee LD_{50}s at 24, 43, and 67 h after exposure. Finally, we examined the effect of mode of application on bee and mite mortality.

Materials and Methods

Chemicals. Carvacrol, camphor, cineole, cinnamon oil, clove oil, citronellal, *d*-limonene, menthol, thymol, and *trans*-anethole were purchased from Sigma (St. Louis, MO). Benzyl acetate, benzyl alcohol, cinnamic alcohol, cinnamic aldehyde, eugenol, methyl salicylate, phenyl ethyl alcohol, phenyl ethyl propionate, pulegone, and α-terpineol were purchased from Arylessence (Marietta, GA). Terpinen-4-ol was purchased from Acros Organics, NJ, and Magic3 is a proprietary mixture of five essential oil constituents prepared by Murray Isman (University of British Columbia, Vancouver, BC, Canada). Technical grade tau-fluvalinate (Wellmark International, Dallas, TX) and 90% formic acid (Fisher, Fair Lawn, NJ) were included as positive controls.

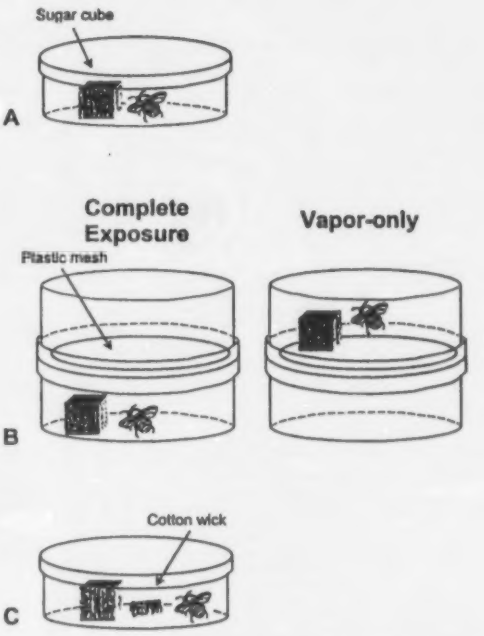

Fig. 1. Bioassay dish designs. (A) Dish design for screening and complete exposure-only experiments. (B) Two-chambered dish for mode of application experiments. (C) Dish for formic acid experiment with cotton wick glued to bottom.

Test Dish Design. Treatments initially were assayed in unmodified disposable plastic petri dishes (60 by 20 mm) (Fig. 1A). New dishes were used in each experiment. Essential oil constituents and tau-fluvalinate were diluted in reagent-grade hexane (with the exception of cinnamic alcohol, which was diluted in methanol) to the desired concentration, and then 1 ml was applied to the bottom of the appropriate dish with a Gilson Pipetman. Dishes were left open until the hexane evaporated; (~15 min at room temperature). With the exception of formic acid, all treatments tested were less volatile than their diluting solvents, as indicated by their high boiling points (Table 1). It is unlikely that a significant amount of any of the test treatments evaporated during this time. Doses were calculated in milligrams applied per cage. A sugar cube then was placed inside for food, and the dishes were closed, randomized, and stacked in holding racks. Individuals placed in these dishes were therefore exposed to topical, vapor, and potentially oral applications of the treatments. For simplicity, we refer to this combination of exposure routes as complete exposure.

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Table 1. Solvent and treatment boiling points

Treatment	Boiling point, °C[a]	Treatment	Boiling point, °C[a]
α-Terpineol	220	Formic acid	101
Benzyl acetate	206	Limonene	176
Benzyl alcohol	205	Magic3[e]	215–222
Camphor	207	Menthol	212
Carvacrol	237	Methyl salicylate	223
Cineole	177	Phenyl ethyl alcohol	204
Cinnamic alcohol	250	Phenyl ethyl propionate[d]	244
Cinnamic aldehyde	251	Pulegone	224
Cinnamon oil[b]	251–253	Tau-fluvalinate[d]	450
Citronellal	208	Terpinen-4-ol[d, e]	220
Clove oil	253	Thymol	233
Eugenol	253	*Trans*-anethole	235
Solvent			
Methanol	65		
n-Hexane	69		
Water	100		

[a] Boiling points at standard pressure. Unless noted, from CRC (1992).
[b] No boiling point available. Value is estimated from the major constituents eugenol and cinnamic aldehyde (Lawless 1995).
[c] Estimated (M. B. Isman, personal communication).
[d] From Chemfinder.com (1999).
[e] Original boiling point (88–90°C at 8 mm Hg) corrected to standard pressure (Hass and Newton 1992).

Dishes for assessing the effect of mode of application (Fig. 1B) were constructed by melting a hole (4.4 cm diameter) in two petri dish lids by using a heated length of copper tubing, which also fused them together. A circle of plastic mesh (5.7 cm diameter; 32 by 32 high-density polyethylene mesh, Synthetic Industries, Lumite Division, Gainsville, GA) then was glued to one side to prevent movement between the two chambers. Treatment preparations were applied to the bottom chamber. Mites and bees were either placed in the bottom chamber and received complete-exposure applications, or were placed in the top chamber and were exposed to vapors only. New dishes were used in each experiment.

Formic acid is highly volatile and water soluble and consequentially was assayed in two separate experiments by using modified versions of both cages (Fig. 1C). A stock solution of 90% formic acid was diluted in distilled water to the desired concentrations, and 0.5 ml was applied to a 2-cm cotton wick glued to the bottom of each cage.

Bees and Mites. Bees and mites were taken from 13 highly infested colonies of *A. mellifera* located in a remote apiary near Simon Fraser University, BC, Canada. All colonies were of mixed race and had been left untreated for *Varroa* for the preceding 12–24 mo. Mite populations were regularly sampled from these colonies with adhesive boards placed in the bottom of hives for 24 h. Bee brood from infested colonies was periodically exchanged with that of uninfected colonies to maintain mite densities in infested colonies below levels that were lethal.

In the screening and formic acid experiments, bioassay dishes were seeded with *Varroa* at the remote apiary, using adult bees obtained from one or two infested colonies. Individual bees were collected from brood frames removed from colonies and were inspected for *Varroa* before being placed in prepared dishes. Despite high infestation levels, it was often difficult and time-consuming to find sufficient *Varroa* for each experiment; consequently, each dish received six bees and a minimum of one mite.

To increase the number of mites per dish and permit a more detailed statistical analysis, newly emerged adult bees were used in the complete-exposure and application-mode experiments. Bees were emerged from 8–12 frames of infested brood placed in nucleus colony boxes held in a heated room (3°C for 5 d). Approximately 50–100 adult bees also were kept in each box to assist and feed the newly emerged bees, however, only newly emerged adults (0–5 d old) were collected for experimental trials. Densities of mites obtained from these bees were substantially higher, allowing bioassay dishes to be seeded with six bees and a minimum of four mites.

After preparation and seeding with mites, bioassay dishes were randomly stacked and stored in Conviron incubators maintained at 30°C and 70% RH.

Observations. Mortality of *A. mellifera* and *V. jacobsoni* in each dish was assessed at ~24, 48, and 72 h. Mortality was assessed by gently agitating each dish so that the sugar cube would brush against any motionless mites and bees; lack of response to this stimulus after 15 s was considered an indication of mortality. Dead bees were visually inspected for the presence of mites. The dishes were frozen after the final observation, and the total number of bees and mites in each dish was verified by immersing the bees in soapy water, squeezing each individual to remove any mites, and recording the total number of each species.

Stage 1—Screening Experiments. Experiments were conducted over five consecutive weeks in May and June 1998. Twenty-two essential oil constituents and the positive controls tau-fluvalinate and formic acid were assayed at three to six doses, each replicated three to six times. Each experiment included solvent and untreated controls.

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Table 2. Ranked best-dose results of screening experiments

Treatment	Doses assayed, mg/cage	Best dose, mg/cage	Best time, h	Avg % bee death ± SE	Avg % mite death ± SE
Tau-fluvalinate[a]	605, 505, 467, 400, 300, 60, 6, 3, 2, 1, 0.6	6	24	0 ± 0	100 ± 0
Clove oil[c]	25, 5, 4, 2, 1, 0.2	1	48	3 ± 3	100 ± 0 ♦
Benzyl acetate	24, 5, 1	5	48	6 ± 6	93 ± 6 ♦
Thymol[c]	10, 2, 0.4, 0.08, 0.016	0.4	48	0 ± 0	83 ± 16 ♦
Carvacrol	5, 1, 0.1	1	24	11 ± 6	100 ± 0 ♦
Methyl salicylate[c]	24, 9.9, 5, 1.98, 1, 0.39	5	24	22 ± 15	100 ± 0 ♦
Formic acid[a,b]	3, 1.5, 0.5, 0.25	1.5	24	28 ± 10	100 ± 0
Eugenol	9.9, 2.5, 0.62, 0.15, 0.04	0.62	24	0 ± 0	42 ± 19
Magic3[d]	9.9, 2.5, 0.62, 0.15, 0.04	0.62	48	6 ± 4	47 ± 18 ♦
Phenyl ethyl propionate	30, 15, 5, 1, 0.1	5	24	39 ± 22	100 ± 0
Cinnamon oil	24, 5, 1	5	24	44 ± 20	100 ± 0
Trans-anethole	5, 1, 0.1	5	24	44 ± 20	100 ± 0
Terpinen-4-ol	9.9, 2.5, 0.62, 0.15, 0.04	2.5	48	6 ± 6	33 ± 20
Menthol	5, 1, 0.1	5	24	56 ± 11	100 ± 0
Pulegone	5, 1, 0.1	5	24	72 ± 20	100 ± 0
Phenyl ethyl alcohol	24, 5, 1	5	24	83 ± 17	100 ± 0
Cinnamic aldehyde	5, 1, 0.1	5	24	94 ± 6	100 ± 0
Citronellal	30, 15, 5, 1, 0.1	15	24	94 ± 6	100 ± 0
α-Terpineol	5, 1, 0.1	5	24	78 ± 15	80 ± 17
Camphor	30, 15, 5, 1, 0.1	None	—	—	—
Limonene	30, 15, 5, 1, 0.1	None	—	—	—
Cineole	30, 15, 5, 1, 0.1	None	—	—	—
Benzyl alcohol	24, 5, 1	None	—	—	—
Cinnamic alcohol	24, 5, 1	None	—	—	—

Treatments selected for further testing indicated with ♦. Deaths of best dose at best time ± standard error.
[a] Products currently registered for *Varroa* control in Canada and the United States.
[b] Formic acid doses are given in percentage concentration of the 0.5 ml applied to the wick.
[c] Essential oils selected for further testing that are exempt from registration in the United States (Quarles 1996).
[d] The major constituents of Magic3 are exempt from registration in the United States (Quarles 1996).

Data Analysis. Mean percentage of *A. mellifera* and *V. jacobsoni* mortality per dish was calculated for each dose, observation time, and treatment. A best-dose and best-observation time was selected for each treatment (Table 2) based on their mite selectivity by using the following criteria, in order of preference: (1) *Varroa* mortality >70% with <30% bee mortality, (2) *Varroa* mortality >40% with <10% bee mortality, (3) *Varroa* mortality greater than bee mortality. No best dose was selected in cases where bee mortality was consistently greater than *Varroa* mortality. Treatments were ranked relative to each other based on their best-dose response (Table 2). Seventy percent *Varroa* mortality was selected because acaricide treatments are considered ineffective unless >70% of mites are killed in field situations (Koeniger and Fuchs 1989). Thirty percent bee mortality was arbitrarily chosen as the maximum tolerable bee mortality given the artificial conditions of the dish environment. Six treatments were selected for further testing: clove oil, benzyl acetate, thymol, carvacrol, methyl salicylate, and Magic3. Magic3 was selected for further testing rather than eugenol, the main constituent of clove oil (Lawless 1995), because we were interested in examining the efficacy of a mixture of essential oil constituents rather than a single active ingredient.

Stage 2—Complete Exposure Only. The six most promising treatments were assayed simultaneously in July 1998 in unmodified petri dishes. Each treatment was tested at seven doses, including zero as a solvent control, replicated five times. Seven doses of tau-fluvalinate were included as a positive control. Treatment doses were selected based on the screening experiments to encompass 0–100% mortality of both species. Formic acid was assayed separately because it required a different dish design. For this treatment, five doses, including 0 mg per cage as a solvent control, were replicated six times. Untreated control treatments were included in both experiments.

Data Analysis. The data set for carvacrol was removed from the analysis because of the accidental contamination of some test dishes during preparation. LD_{50} values and inverse 95% fiducial confidence limits (CL) of *A. mellifera* and *V. jacobsoni* were estimated for the remaining treatments at each observation time by using logistic regression (SAS Institute 1997). For this analysis, the model was mortality = dose, with dose as a continuous variable and mortality as a binary response. Logistic regression was used because it best suited our data set and experimental design (Finney 1971). The LD_{50}s with 95% CL that did not overlap were considered significantly different. This criterion was used to examine whether or not the LD_{50} estimates for each species changed significantly with time. A selectivity ratio (SR) (*A. mellifera* LD_{50}/*V. jacobsoni* LD_{50}) also was calculated for each treatment at each observation time. Ratios were considered to be meaningful indicators of selectivity only if the 95% CL of the LD_{50}s being compared did not overlap.

Stage 3—Mode of Application. Three treatments (Magic3, thymol, and clove oil) were tested in August 1998. Complete exposure and vapor applications of

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each treatment were tested at six doses, including 0 mg per cage as a solvent control, replicated five times. Eight doses of vapor applications of formic acid, replicated three times, also were tested.

Data Analysis. LD_{50} values and inverse 95% fiducial confidence limits were estimated by treatment, application method (vapor-only or complete exposure), species, and observation time as described.

Results

Stage 1 –Screening. Tau-fluvalinate, a known acaricide, had the highest ranked best-dose response (Table 2). Clove oil, benzyl acetate, thymol, carvacrol, and methyl salicylate had best-dose responses that were comparable with or better than formic acid. Thymol was toxic to mites at the lowest best dose followed by eugenol and Magic3. All treatments tested killed >90% of bees at their top dose with the exception of limonene, which was minimally toxic to bees (<30%) and nontoxic to mites at all doses assayed.

Stage 2 –Complete Exposure Only. The estimated LD_{50} values and selectivity ratios for each treatment at each time interval are shown in Fig. 2. Regression statistics (*n*, slope, standard error) and the doses applied are shown in Table 3.

Selectivity. Thymol at 43 h was the most selective test treatment (SR = 4.3) and benzyl acetate was the least selective (SR = 1.4 at 43 h) (Fig. 2). Tau-fluvalinate was >200 times more selective than thymol and >260 times more selective than our best estimates for formic acid, our second known control. The selectivity ratios for benzyl acetate at 24 and 67 h were not considered meaningful becaaue of the overlapping *A. mellifera* and *V. jcobsoni* 95% CL. The large, asymmetrical confidence limits calculated for formic acid mite LD_{50} estimates are caaused by the small number of mites (n = 40) and limited dose range assayed (Robertson and Preisler 1992) and are not indicative of the variation in the data set. Given the results of the mode of application experiment (below), it seemed reasonable to estimate the selectivity ratio of formic acid to be between 2 and 3. Therefore, thymol, clove oil, Magic3, and methyl salicylate demonstrated selectivity equal to or greater than our best estimate for formic acid.

Effects of Time. Magic3 and formic acid showed no significant change in *A. mellifera* or *V. jacobsoni* LD_{50} estimates over the three observation times (Fig. 2). Methyl salicylate showed no significant changes in *V. jacobsoni* LD_{50}, and *A. mellifera* estimates did not decrease significantly until 67 h. Clove oil LD_{50} estimates for both species decreased with time, resulting in a large selectivity ratio decrease of 0.94 (27%) between 24 and 43 h, and a smaller decreasc at 67 h. Benzyl acetate showed minor variation in LD_{50} estimates for both species with time, and displayed nonoverlapping confidence limits only at 43 h. Thymol's selectivity ratio increased 1.3 (43%) between 24 and 43 h, the largest relative change of any treatment, as a result of its *V. jacobsoni* LD_{50} estimate decreasing by almost 50% in that time interval. Thymol's selectivity ratio

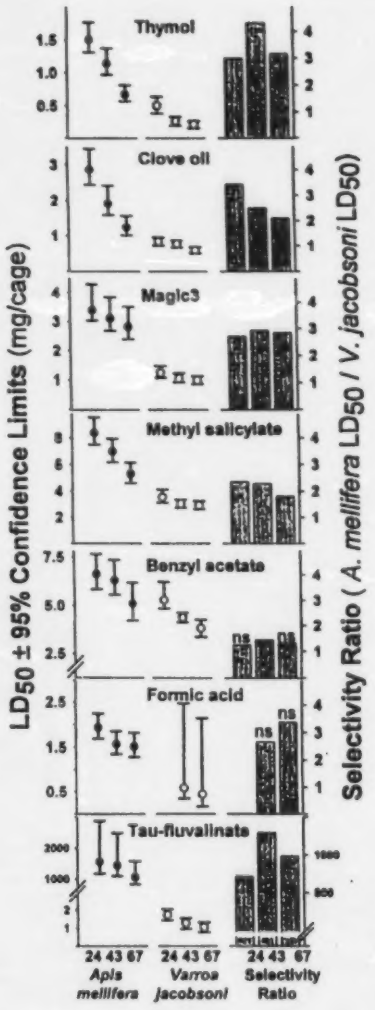

Fig. 2. Estimated LD_{50} values and selectivity ratios for complete exposure-only experiment. Regression statistics (*n*, slope, SE) are given in Table 3. Ratios marked ns are not considered meaningful because of overlapping *A. mellifera* and *V. jacobsoni* LD_{50} 95% CL.

then decreased by a large margin (26%) at 67 h. Tau-fluvalinate's estimates for *A. mellifera* did not change significantly with time, although its *V. jacobsoni* LD_{50} estimate decreased significantly at 67 h. Tau-fluvalinate's confidence limit for *A. mellifera* showed the largest variation (>100%) because of low and irregular bee mortality at all the doses assayed. For this reason, the changes in tau-fluvalinate's selectivity ratio were not considered a good indication of the effects of time.

Stage 3–Mode of Application. The estimated LD_{50} values for *V. jacobsoni* with each treatment at each

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Table 3. Logistic regression statistics for contact-only experiment

Treatment and doses applied, mg/cage	Time, h	*Apis mellifera*		*Varroa jacobsoni*	
		n	Slope ± SE	n	Slope ± SE
Thymol	24	210	2.47 ± 0.34	173	3.15 ± 0.57
0.00, 0.16, 0.32, 0.41,	43		2.42 ± 0.33		6.90 ± 1.27
0.59, 1.18, 2.06	67		3.86 ± 0.58		9.32 ± 1.65
Clove oil	24	210	1.24 ± 0.17	193	3.56 ± 0.52
0.00, 0.31, 0.57, 0.92,	43		1.39 ± 0.23		4.73 ± 0.66
1.19, 1.49, 3.98	67		1.45 ± 0.27		5.16 ± 0.77
Magic3	24	208	2.17 ± 0.62	168	2.90 ± 0.49
0.00, 0.25, 0.50, 0.67,	43		1.38 ± 0.24		3.60 ± 0.58
1.00, 1.51, 3.02	67		1.24 ± 0.20		4.23 ± 0.68
Methyl salicylate	24	210	0.49 ± 0.07	161	1.20 ± 0.24
0.00, 1.00, 1.98, 2.97,	43		0.52 ± 0.06		2.22 ± 0.44
3.96, 7.92, 9.9	67		0.60 ± 0.07		2.05 ± 0.39
Benzyl acetate	24	210	0.79 ± 0.12	200	1.42 ± 0.38
0.00, 0.99, 1.98, 3.96,	43		0.68 ± 0.10		2.86 ± 0.61
5.05, 9.00, 15.15	67		0.40 ± 0.06		1.18 ± 0.19
Tau-fluvalinate	24	234	0.0023 ± 0.0006	191	2.14 ± 0.42
0.00, 1.00, 2.00, 3.01,	43		0.0021 ± 0.0005		2.30 ± 0.44
501, 605, 805, 1003	67		0.0019 ± 0.0004		2.78 ± 0.58
Formic acid	24	181	2.34 ± 0.34	40	n/a
0.00, 0.25, 0.50, 1.50, 3.00	47		2.40 ± 0.35		3.82 ± 1.68
	71		2.02 ± 0.28		3.56 ± 1.63

Time is time of observation (length of exposure in hours); n, number of insects tested; n/a, not available due to insufficient data. The LD_{50} values and 95% CL were calculated using logistic regression and are shown in Fig. 2. All regressions gave chi-square values that were significant at $P < 0.05$.

F3 T4 time interval are shown in Fig. 3. Regression statistics (n, slope, standard error) and the doses applied are shown in Table 4.

(1) Selectivity. *A. mellifera* LD_{50} estimates were not available for clove oil and thymol because of low bee mortality at all doses assayed. For Magic3 the estimated LD_{50} for *A. mellifera* at 43 h was 3.8 mg per cage for the complete exposure application ($n = 180, 0.65 \pm 0.10$, $P < 0.001$) and 4.9 mg per cage for the vapor application ($n = 180, 0.36 \pm 0.08$, $P < 0.001$), resulting in selectivity ratios of 2.1 and 2.2, respectively. The selectivity ratio of formic acid maintained a constant value of (~2.1 throughout the experiment. The mite LD_{50} values of formic acid were predicted with much tighter confidence intervals than in the complete exposure-only experiment as a result of the wider range of doses tested.

Effect of Mode of Application. Vapor and complete-exposure application toxicity may be compared by examining the *V. jacobsoni* LD_{50} estimates of each treatment at each time interval. For thymol, complete-exposure and vapor LD_{50} estimates did not change significantly with time, and complete-exposure estimates were significantly lower at all time intervals, as indicated by their nonoverlapping 95% CL. For Magic3, both complete exposure and vapor LD_{50} estimates decreased with time, and complete-exposure estimates were significantly lower than vapor estimates at all time intervals. For clove oil, both complete-exposure and vapor LD_{50} estimates decreased with time, and complete-exposure and vapor 95% CL overlapped at all intervals.

Toxicity. The complete-exposure LD_{50} estimates for thymol and Magic3 from the mode of application experiment, using the two-chambered dish, were significantly higher (nonoverlapping confidence limits) than those obtained in the single-chamber experiment. On average, thymol and Magic3 were five and three times more toxic, respectively, in the single-chamber dish than in the two-chambered dish. The clove oil LD_{50} estimates of both experiments had overlapping 95% CL.

Discussion

The bioassay presented herein provides a simple, versatile, inexpensive, and rapid mechanism for screening new miticide treatments. Testing bees and mites simultaneously allows for the determination of selectivity ratios, and positive controls provide standards for comparison. Estimated LD_{50} values with 95% CL provide a convenient means of examining the effects of mode of application and time. However, this analysis of our data has two important limitations, as follows: (1) estimated selectivity ratios do not have confidence limits associated with them and therefore must be interpreted in a general fashion, and (2) observations at each exposure time were not independent and may have been confounded by other factors, such as the vapor release rates of each treatment and their mode of action (vapor, oral, or topical). Future experiments should estimate the vapor release rate of treatments of interest under experimental conditions, use separate cages for each desired exposure time, and, if desired, perform replicate experiments to determine the confidence limits of the selectivity ratios.

It is important to realize that there is no easy way to determine the exact test treatment concentration in each dish at any one time. Vapor release rates could be determined and used to estimate what proportion of each treatment was in gaseous or solid form during each observation period; however, this estimate would

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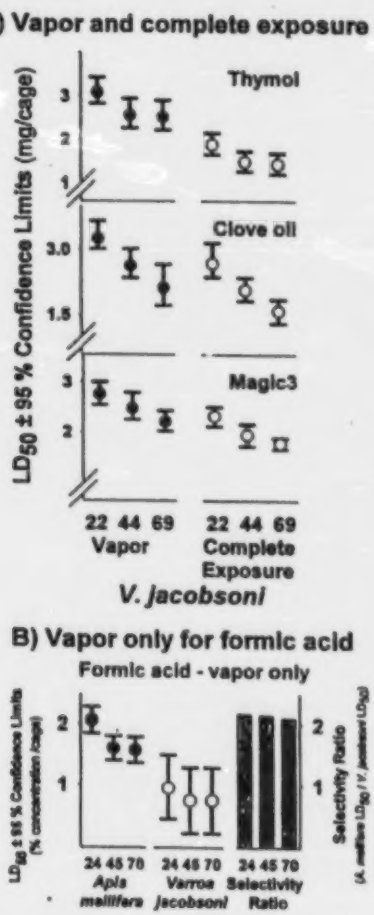

Fig. 3. Estimated LD_{50} values for mode of application experiments. Regression statistics (*n*, slope, SE) are given in Table 4. (A) *V. jacobsoni* estimated LD_{50} 95% CL for vapor and complete exposure applications of thymol, Magic3, and clove oil at 22, 44, and 69 h. *A. mellifera* estimates were not available because of low bee mortality at all doses assayed. (B) The LD_{50} estimates for *V. jacobsoni* and *A. mellifera* for formic acid, using a modified two-chambered dish design.

not account for the amount ingested by or accumulated on each bee or mite. Additionally, some test treatment vapor may have escaped from dishes during their preparation or seeding, or through the small airspace between the base and lid of the dish. This leaky dish design was intentionally adopted to reduce the accumulation of vapors in the dish and to mimic the somewhat porous conditions in the hive. Therefore, although the graded responses obtained provide useful information, they are not true estimates of topical lethal dose or gaseous lethal concentration values for either species. This limitation of our assay is outweighed by its ability to simultaneously test multiple

Table 4. Logistic regression statistics for mode of application experiment

Treatment and doses applied, mg/cage	Mode of appl.*	Time, h	*Varroa jacobsoni*	
			n	Slope ± SE
Thymol	V	22	153	2.68 ± 0.65
0.00, 0.27, 0.52,	V	44		1.78 ± 0.29
1.05, 2.09, 3.22	V	69		1.77 ± 0.28
	C	22	159	2.40 ± 0.36
	C	44		2.45 ± 0.35
	C	69		2.40 ± 0.34
Clove oil	V	22	186	2.24 ± 0.43
0.00, 0.89, 1.45,	V	44		1.37 ± 0.22
1.94, 2.49, 3.32	V	69		0.79 ± 0.16
	C	22	198	1.16 ± 0.20
	C	44		1.43 ± 0.21
	C	69		1.38 ± 0.19
Magic3	V	22	187	2.85 ± 0.49
0.00, 1.43, 1.90,	V	44		2.64 ± 0.41
3.80, 2.85, 6.14	V	69		3.06 ± 0.50
	C	22	197	3.28 ± 0.50
	C	44		2.46 ± 0.40
	C	69		7.42 ± 1.76
Formic acid	V	24	40	4.70 ± 1.91
0.0, 0.5, 1.0, 1.5, 2.0,	V	45		6.23 ± 2.67
2.5, 3.0, 4.0, 4.5	V	70		6.23 ± 2.67
				Apis mellifera
Formic acid	V	24	162	2.75 ± 0.46
0.0, 0.5, 1.0, 1.5, 2.0,	V	45		3.08 ± 0.52
2.5, 3.0, 4.0, 4.5	V	70		2.71 ± 0.44

Time is time of observation (length of exposure in hours); *n*, number of insects tested. The LD_{50} values and 95% CL were calculated using logistic regression and are shown in Fig. 3. All regressions gave chi-square values that were significant at $P < 0.05$.

* V, vapor only; C, complete exposure.

species and modes of application in a rapid and inexpensive manner.

The screening experiments indicated that clove oil, benzyl acetate, thymol, carvacrol, methyl salicylate, eugenol, and Magic3 are mite-selective at one or more doses. Other assays have reported mite selectivity with wintergreen (methyl salicylate), clove oil, eugenol, and thymol (Hoppe and Ritter 1989, Kraus et al. 1994, Imdorf et al. 1995b) However, Imdorf et al. (1995b) also had excellent results with menthol and camphor, which demonstrated poor mite selectivity in our screening process (Table 2). This discrepancy may be because our protocol tested a combination of topical and vapor applications, whereas their experiment tested vapor-only applications. This result suggests that menthol and camphor may have topical insecticidal properties that are harmful to bees, and presents the possibility that some of the treatments we screened may have demonstrated greater selectivity if tested with a vapor-only application. Consequentially, we recommend future testing and screening of treatments using our two-chambered dish design to compare both complete-exposure and vapor application methods.

The complete exposure experiments demonstrated that tau-fluvalinate is highly selective, with a selectivity ratio that was consistently two orders of magnitude greater than that of any other treatment tested, in-

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cluding formic acid. The low selectivity of these natural products may explain why they have had variable field efficacy, and provides a challenge to developing practical application methods. However, thymol, clove oil, Magic3, and methyl salicylate all demonstrated selectivity that was equal to or greater than that of formic acid, a registered and currently used *Varroa* control agent, indicating that they do have a level of selectivity acceptable to beekeepers. Clove oil, methyl salicylate (wintergreen), thyme oil, and the main constituents of Magic3 have been exempted from registration by the Environmental Protection Agency in the United States since 1996 (Quarles 1996); therefore, manufacturers are free to sell these materials as pesticides without undergoing the costly and time-consuming registration process.

Our application mode experiments indicated that both topical and vapor applications of essential oils have insecticidal properties. All three treatments tested at this stage and formic acid were miticidal when applied as vapors, which may explain their temperature-dependent effects. An application device that released a consistent, temperature-independent amount of vapor could circumvent this problem. Estimated *V. jacobsoni* LD_{50} values for thymol and Magic3 complete-exposure applications were significantly lower than those of vapor-only applications, indicating that topical or oral exposure to these treatments increases mite mortality. There were no significant differences between the complete-exposure and vapor-only applications of clove oil, although complete-exposure *V. jacobsoni* LD_{50} values were lower at all observation times, suggesting that topical and oral exposure to clove oil has little or no effect on mite mortality.

The complete exposure toxicity of thymol and Magic3 decreased by five and three times respectively in the two-chambered dish relative to the single chamber dish, whereas the clove oil LD_{50} estimates of the two dish designs were not significantly different. In terms of exposure, the only difference between the two designs was a doubling of the air volume in which vapors were dispersed; the surface area of topical exposure remained the same. Differing dose–responses may have been caused by differences in the physiological mode of action of each treatment, a nonlinear relationship between vapor pressure and volume, increased airflow in the larger cage, a chemical interaction with the plastic mesh, or a combination of these factors. Future experiments should examine a broader dose range to determine the effect of application mode on bee mortality and treatment selectivity. Understanding the effect of mode of application would allow better design of application devices that optimize the selectivity of each treatment.

Varying the amount of time that subjects are exposed to each treatment also may effect treatment selectivity. Our results suggest that some treatments, such as clove oil, may be most effective when applied at high concentrations for short time periods, whereas other treatments, such as thymol, may require slow release to optimize their efficiency.

Essential oil constituents also may have sublethal effects on *Varroa*. Essential oil constituents such as thymol, menthol, and camphor inhibit the feeding, growth, and reproduction of a variety of insect and mite pests (Larson and Berry 1984, Mansour et al. 1986, Cook 1992, Rice and Coats 1994, Lee et al. 1997). Colin et al. (1990) and Kraus et al. (1994) have studied the repellent effects of essential oils on *Varroa*, and Colin et al. (1990) hypothesized that long-term repellency may reduce *Varroa* mite fecundity. A small reduction in fecundity may have a large impact on the growth of the population because of *Varroa*'s low reproductive rate (Yoshida and Fuchs 1989). Essential oils also provide some measure of control over *Acarapis woodi* (Rennie), another bee parasite (Calderone et al. 1991, 1997; Ellis and Baxendale 1997), and with some modifications our laboratory bioassay could be used to test for tracheal mite selectivity (A.P.M, unpublished data).

Tau-fluvalinate is highly selective and easy to use, providing advantages over essential oils for *Varroa* control. Unfortunately, its widespread use has resulted in the development of mite resistance (Elzen et al. 1998) and the accumulation of residues in honey and wax (Wallner 1995). Although it is possible that another highly selective and potentially registrable product such as tau-fluvalinate will be discovered in the future, it seems more likely that beekeepers will have to learn to work with less selective treatments. Natural products with low selectivity such as formic acid and the essential oil constituents tested here can be effective *Varroa* control agents. We currently are working to develop slow-release, temperature-independent application methods for thymol and Magic3 that would increase selectivity and minimize the amount of labor required to apply treatments. We also are experimenting with using essential oil constituents in combination with hygienic strains of bees (Spivak 1996) and kairomone based *Varroa* traps (Trouiller et al. 1992, 1994). An IPM program incorporating these and other elements have the potential to provide a cost-effective solution for *Varroa* management. AQ: A

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